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Application No. 2003/0760

Date of Filing 14 October 2003

Applicant THE PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED TRINITY OF QUEEN ELIZABETH, NEAR DUBLIN, a registered charity of College Green, Dublin 2, Ireland.

Dated this 19 day of October 2004.

An officer authorised by the
Controller of Patents, Designs and Trademarks.

030760

FORM NO. 1

# REQUEST FOR THE GRANT OF A PATENT

## PATENTS ACT, 1992

The Applicant(s) named herein hereby request(s)

X the grant of a patent under Part II of the Act

_____ the grant of a short-term patent under Part III of the Act on the basis of the information furnished hereunder.

1. Applicant(s)

Name: THE PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED TRINITY OF QUEEN ELIZABETH, NEAR DUBLIN

Address: College Green, Dublin 2, Ireland

Description/Nationality

A registered charity

2. Title of Invention

"A method"

3. Declaration of Priority on basis of previously filed application(s) for same invention (Sections 25 & 26)

| Previous filing date | Country in or for which filed | Filing No. |
|---|---|---|

4. Identification of Inventor(s)
Name(s) of person(s) believed by Applicants(s) to be the inventor(s)

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Statement of right to be granted a patent (Section 17(2) (b))

The Applicant derives the rights to the Invention by virtue of a Deed of Assignment dated October 13, 2003.

6. Items accompanying this Request – tick as appropriate

| | | |
|---|---|---|
| (i) | X | Prescribed filing fee (€125.00) |
| (ii) | X | Specification containing a description and claims |
| | | Specification containing a description only |
| | X | Drawings referred to in description or claims |
| (iii) | | An abstract |
| (iv) | | Copy of previous application(s) whose priority is claimed |
| (v) | | Translation of previous application(s) whose priority is claimed |
| (vi) | X | Authorisation of Agent (this may be given at 8 below if this Request is signed by the Applicant (s)) |

7. Divisional Application (s)

The following information is applicable to the present application which is made under Section 24 –

Earlier Application No: .....

Filing Date: .....

8. Agent

The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

| Name | Address |
|---|---|
| John A. O'Brien & Associates | The address recorded for the time being in the Register of Patent Agents, and currently Third Floor, Duncairn House, 14 Carysfort Avenue, Blackrock, Co. Dublin, Ireland. |

9. Address for Service (if different from that at 8)

As above

Signed _____ JOHN A. O'BRIEN & ASSOCIATES

Date October 14, 2003

# A method

## Introduction

The invention relates to adenylate cyclase toxin (CyaA) or a derivative or mutant or fragment or variant or peptide thereof.

Adenylate cyclase toxin (CyaA) is a virulence factor of the Gram-negative bacteria, *B. pertussis*, that causes the respiratory disease whooping cough. Bacteria deficient in CyaA are less pathogenic in mice and CyaA has been shown to subvert immune responses to *B. pertussis* by interfering with chemotaxis, phagocytosis and superoxide production in host cells, through the generation of supraphysiological levels of cAMP. Furthermore, CyaA causes lysis and cytotoxicity in a variety of cells and causes apoptosis in macrophages. CyaA is encoded by the *cyaA* gene and is post-translationally activated through palmitoylation of $K_{983}$ by the product of the *cyaC* gene. The C-terminal 1306 amino acids contain a series of nonapeptide repeats involved in calcium binding similar to the repeat in the toxin (RTX) family of exotoxins which have haemolytic and immune stimulatory ability. Palmitoylation of CyaA by its accessory protein, CyaC, is necessary for binding to and haemolysis of sheep red blood cells and for its ability to lyse macrophages and T cells (1, 2). The N-terminal 400 amino acids contain the catalytic domain that converts ATP to cAMP. Upon cell binding the enzymatic domain is delivered into the cytosol where it must bind eukaryotic calmodulin to become enzymatically active.

The invasive nature of CyaA has been employed to deliver antigenic peptides to the endogenous route of antigen processing for presentation to MHC class I-restricted $CD8^+$ T cells (3). Recently it has been shown that an enzymatically inactive CyaA could deliver an epitope into the MHC class II processing pathway for activation of $CD4^+$ cells (4). In addition, CyaA has been shown to enhance antibody levels to co-administered ovalbumin (5). This study also suggested that a non-active form of

CyaA expressed in *E. coli* in the absence of the *cyaC* gene, which was non-invasive and lacked haemolytic and cytotoxic activity, had limited adjuvant activity for antibody response, when compared with the active toxin (5). CyaA has also been shown to promote Th1 responses to an expressed viral epitope (6). The adjuvant activity of CyaA may reside in its ability to activate cells of the innate immune system through the upregulation of cAMP (7) and/or the binding to the CD11b/CD18 $\alpha_M\beta_2$ integrin (8), expressed on innate immune cells, including macrophages and dendritic cells (DC).

Cells of the innate immune system, especially DC, direct the differentiation of naïve $CD4^+$ T cells into functionally distinct Th1, Th2 or regulatory T (Tr) cell subtypes. Activation of immature DC though binding of conserved microbial molecules to pathogen recognition receptors (PRRs), such as Toll-like receptors (TLR) and integrins, is accompanied by maturation and homing to the lymph nodes, where the mature DC presents antigen to the naïve T cells. Activation of DC by pathogen derived molecules plays a critical role in regulating the differentiation of naïve $CD4^+$ T cells into distinct T cell subtypes (10, 11, 12). Th1 cells confer protection against intracellular infection but are also associated with inflammatory responses and autoimmune disease, whereas Th2 cells are involved in allergic responses. Tr cells are capable of suppressing Th1 and Th2 responses (10, 11, 12).

It is clear that any method of modulating inflammatory activity or inducing regulatory T cells in vivo would be of valuable therapeutic benefit.

## Statements of Invention

According to the invention there is provided a method for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides a method for the treatment and/or prophylaxis of an immune-mediated disorder comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention further provides a method for the treatment and/or prophylaxis of an autoimmune disease comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

In one embodiment of the invention the adenylate cyclase toxin (CyaA) is combined with self or foreign antigens or peptides thereof.

Preferably the adenylate cyclase toxin (CyaA) is derived from *Bordetella pertussis*, *Bordetella bronchisepetica* or *Bordetella parapertussis*.

In one embodiment of the invention the agent modulates inflammatory cytokine production.

In another embodiment of the invention the immunomodulatory effects of CyaA on cells of the innate immune system is dependent on co-activation with a Toll-like receptor (TLR) ligand. Preferably the Toll-like receptor (TLR) ligand is LPS or other toll-like receptor ligands, including but not confined to CpG motifs, dsRNA, Poly (I:C).

In one aspect of the invention CyaA promotes IL-10 and IL-6 production by macrophages and dendritic cells (DC).

In one embodiment of the invention the CyaA synergises with LPS to promote IL-10 and IL-6 production by macrophages and dendritic cells (DC).

In another embodiment of the invention the A method as claimed in any preceding claim wherein CyaA inhibits inflammatory cytokines, chemokines or other inflammatory mediators. Preferably the inflammatory cytokine is selected from any one or more of IL-12 or TNF-$\alpha$, IFN-$\gamma$, IL-1, IL-23 or IL-27. The inflammatory chemokine may be macrophage inflammatory protein-1$\alpha$ or macrophage inflammatory protein-1$\beta$.

In one embodiment of the invention CyaA promotes dendritic cell maturation following co-activation with TLR-ligands. Preferably CyaA promotes CD80 expression by dendritic cells.

In another embodiment of the invention CyaA inhibits TLR-ligand-induced dendritic cell activation. CyaA may inhibit CD40 and ICAM-1 expression.

In one embodiment of the invention the CyaA acts as an adjuvant *in vivo* to promote the induction of Th2 or Tr cells to co-administered antigens.

In another embodiment of the invention CyaA acts as an adjuvant *in vivo* to promote IgG1 antibodies to co-administered antigens.

CyaA may be present in a non-palmitoylated form.

In one embodiment of the invention the CyaA is in the form of an adjuvant, immunotherapeutic of anti-inflammatory agent.

In one embodiment of the invention the agent modulates inflammatory cytokine production induced by infection or trauma.

The disorder may be sepsis or acute inflammation induced by infection, trauma or injury. The disorder may also be selected from any one or more of Crohn's disease, inflammatory bowel disease, multiple sclerosis, type 1 diabetes or rheumatoid arthritis. The disorder may be asthma or atopic disease.

In one embodiment of the invention the agent is in a form for oral, intranasal, intravenous, intradermal, subcutaneous or intramuscular administration.

The invention provides a pharmaceutical composition comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides an immunomodulator comprising adenylate cyclase toxin (CyaA).

The invention further provides a recombinant non-acylated CyaA having immunomodulatory effects.

The invention also provides a vaccine comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides antibodies to adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention further provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide or product of cells activated by the agent for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder.

The invention provides ause of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or variant or peptide or product of cells activated by the agent

for the prophylaxis and/or treatment of diseases or conditions involving Toll-like receptor (TLR) dependant signalling.

The invention also provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide or product of cells activated by the agent for the prophylaxis and/or treatment of asthma or allergy.

The term derivative or mutant or fragment or variant or peptide as used herein are understood to include any molecule or macromolecule consisting of a functional portion of acylated or non-acylated CyaA.

The term antigen is taken to mean any substance that binds specifically to an antibody or T cell receptor. The term self- or auto-antigen is taken to mean an endogenous antigen on self-tissue in the body, which is not foreign. The term foreign antigen is taken to mean an antigen from a pathogen (bacteria, virus or parasite).

Brief Description of the Invention

The invention will be more clearly understood from the following description thereof with reference to the accompanying drawings in which: -

Fig. 1 are graphs showing the levels of IFN-γ, IL-4, IL-5 and IL-10 produced by lymph node cells from mice after immunization with PBS, KLH alone or with CyaA. BALB/c mice were immunized s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CyaA (1 μg). After 7 days mice were sacrificed and popliteal lymph node cells prepared and stimulated with KLH (2-50 μg/ml) or medium only. After 3 days supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Proliferation was assayed on day 4 by $^{3}H$-Thymidine incorporation. Results represent means (+ SD) of 5 mice per group and are representative of 3 experiments. *, $P < 0.05$; ***, $P < 0.001$ KLH versus KLH + CyaA;

Fig. 2 are graphs showing IL-4, IL-5, IL-10 and IFN-γ production by T cell lines and clones generated from mice immunized with KLH in the presence of CyaA. A) $CD4^+$ T cell lines were generated from lymph nodes of 10 individual mice immunized with KLH and CyaA. B) T cell line 7.2 was cloned by limiting dilution. T cell lines or clones were stimulated with KLH (50 μg/ml) in the presence of autologous APC and cytokine concentrations tested in the supernatants after 3 days, IFN-γ was at background levels in each of the T cell clones;

Fig. 3 is a graph showing antigen-specific IgG, IgG1 and IgG2a levels in mice after immunisation s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CyaA (1 μg) and boosted 21 days later. Serum samples were taken 7 days after one (A) or two (B) immunizations and KLH-specific IgG, IgG1 and IgG2a titres were determined by ELISA. Results are mean (+ SD) titres for 5 mice per group and are representative of 2 experiments. ***, $P < 0.001$ KLH versus KLH + CyaA;

Fig. 4 are graphs showing IL-10, IL-6 and TNF-α levels produced by macrophages incubated with LPS, CyaA, lipopolysaccharide (LPS) and CyaA or CyaA in the presence or absence of polymyxin B. CyaA enhances LPS-induced anti-inflammatory cytokines and suppresses pro-inflammatory cytokines by macrophages. J774 macrophages ($1x10^6$/ml) were incubated with the indicated concentrations of LPS (0-1000 ng/ml), CyaA (1 μg/ml), in presence or absence of polymyxin B (PB; 10 μg/ml). Supernatants were collected at the indicated times and were tested for IL-10, IL-6 and TNF-α by immunoassay. Results are means (+ SD) of triplicate assays and are representative of 3 experiments. **, $P < 0.01$; ***, $P < 0.001$ versus CyaA; ++, $P < 0.01$, +++, $P < 0.001$; versus LPS alone at the same concentration;

Fig. 5 are graphs showing IL-10, IL-6, TNF-$\alpha$ and IL-12p70 levels in dendritic cells (DC) following incubation with LPS, CyaA LPS and CyaA or CyaA in the presence or absence of polymyxin-B. CyaA enhances LPS-induced anti-inflammatory cytokines and suppresses LPS-induced pro-inflammatory cytokines from DC. Murine bone marrow-derived immature DC ($1x10^6$/ml) were incubated with the indicated concentrations of LPS (0-1000 ng/ml), CyaA (1 μg/ml) in presence or absence of polymyxin-B (PB; 10 μg/ml). Supernatants were collected at the indicated times and tested for IL-10, IL-6, TNF-$\alpha$ and IL-12p70 by immunoassay. Results are means (±SD) of triplicate assays and are representative of 3 experiments. **, $P < 0.01$; ***, $P < 0.001$ versus CyaA; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.01$ versus LPS alone at the same concentration;

Fig. 6 are immunofluorecence graphs showing the CD80, CD86, MHC-II, CD40 and ICAM-I expression on DC. CyaA enhances CD80, CD86 and MHC-II, but inhibits CD40 and ICAM-I expression on DC. DC were stimulated with CyaA (1 μg/ml) in the presence of polymyxin B (10 μg/ml), LPS (1 μg/ml), CyaA and LPS or medium only. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86, MHC-II, CD-40 and ICAM-I or with isotype matched controls. Results of immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC. Profiles are shown for a single experiment and are representative of 2 separate experiments;

Fig. 7 are graphs showing cytokine production by lymph node cells from immunized TLR4-defective mice. Bone marrow derived DC from C3H/HeN or C3H/HeJ mice ($1x10^6$/ml) were cultured with the indicated concentrations of LPS (0-10 ng/ml), CyaA (1 μg/ml), in presence or absence of polymyxin-B (PB; 10 μg/ml). Supernatants collected and tested by immunoassay for IL-10 and MIP1-$\alpha$ (4 h) and IL-12p70 and TNF-$\alpha$ (24 h). Results are means

(±SD) of triplicate assays and are representative of 3 experiments. ++, $P <$ 0.01 versus CyaA; $P < 0.001$ versus LPS alone at the same concentration;

Fig. 8 are immunofluorescence graphs showing that CyaA-induced DC activation is altered in TLR4-defective mice. Bone marrow derived DC from C3H/HeN (A) or C3H/HeJ (B) mice ($1x10^6$/ml) were cultured with CyaA (1 μg/ml) either alone or with polymyxin B (PB; 10 μg/ml) or LPS (10 ng/ml) or with medium. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86, MHC-II, CD-40 and ICAM-I or with isotype matched control antibodies. Immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC. The numbers on the right of each histogram refer to the mean florescence intensity of the treated cells, the value for cells treated with medium only is shown on the left of the first histogram in each case. Profiles are shown for a single experiment and are representative of 3 experiments;

Fig. 9 is a graph showing the percentage haemolysis of red blood cells (RBC) under various conditions. A) 100 μl of $5x10^8$ RBC/ml were treated with the indicated amounts of non-palmitoylated-CyaA (NP-CyaA) or palmitoylated CyaA (P-CyaA) for 16 h. 50 il of supernatant was collected and the absorbance at $OD_{541}$ measured and used to calculate the percentage haemolysis. B) 200 μl of $1x10^6$ J774 macrophages/ml were treated with the indicated amounts of NP-CyaA or P-CyaA for 6 h. 50 μl of supernatant was assayed for LDH activity to calculate the percentage cell lysis. Results were compared by the one-way ANOVA with Tukey post-test. $*P<0.05$, $**P<0.01$, $***$ $P<0.001$: treated sample versus medium. Results are means ± SD of a representative experiment performed three times in triplicate;

Fig. 10 are graphs showing the modulation by palmitoylated and non-palmitoylated CyaA of IL-10, TNF-α or MIP-1α production by

macrophages. 1 μg/ml of NP-CyaA or P-CyaA was added to 200 μl of 1 x $10^6$ J774 macrophages/ml in the presence or absence of 10 μg/ml polymyxin B. 2 h later 1 μg/ml LPS was added to the appropriate wells. After a further 4 h incubation supernatants were collected and cytokine concentrations quantified by ELISA. Results were compared by the one-way ANOVA with Tukey post-test. *$P$<0.05, **$P$<0.01, *** $P$<0.001: treated sample versus LPS. Results are means ± SD of a representative experiment performed three times in triplicate;

Fig. 11 are graphs showing the modulation of IL-10 by palmitoylated and non-palmitoylated CyaA as in Fig. 10 over a range of concentrations of palmitoylated and non-palmitoylated CyA. J774 macrophages were treated as in Fig. 10 with the doses of P-CyaA and NP-CyaA as indicated (μg/ml) and 10 ng/ml LPS. Results were compared by the one-way ANOVA with Tukey post-test. *$P$<0.05, **$P$<0.01, *** $P$<0.001: treated sample versus LPS. +$P$<0.05, ++$P$<0.01, +++$P$<0.001: P-CyaA sample versus NP-CyaA at same concentration. Results are means ± SD of a representative experiment performed twice in triplicate;

Fig. 12 are graphs showing the modulation by palmitoylated and non-palmitoylated CyaA of cytokine and chemokine production by DC. The experiment was performed and analysed as described in Figure 10, except with 10 ng/ml LPS and using C3H/HeN and C3H/HeJ BMDC. IL-12 p70 was measured in supernatants following 24 h incubation with toxin. Results were compared by the one-way ANOVA with Tukey post-test. *$P$<0.05, **$P$<0.01, *** $P$<0.001: treated sample versus LPS. Results are means ± SD of a representative experiment performed twice in triplicate;

Fig. 13 are immunofluoresence results showing the stimulation of DC by palmitoylated CyaA (P-CyaA) and non-palmitoylated CyaA (NP-CyaA).

BMDC were treated with 1 ìg/ml NP-CyaA or P-CyaA in the presence or absence of 10 μg/ml polymyxin B. 2 h later 1 μg/ml LPS was added to the appropriate cultures. 24 h later cells were harvested for FACS analysis. Data was acquired with a FACS Calibur (Becton Dickinson) and analysed using CellQuest software. Grey infilled plots represent untreated cells. Black lines indicate treated samples. Results are representative of 2 experiments;

Fig. 14 are graphs of the modulation of cytokine levels by palmitoylated and non-palmitoylated CyaA in macrophages and DC in response to a TLR9 ligand. C3H/HeJ and C3H/HeN DC (A) or J774 macrophages (B) were cultured (1 x $10^6$ /ml) with NP-CyaA or P-CyaA (1 μg/ml) in the presence or absence of polymyxin B (10 μg/ml). After 2 h CpG oligodeoxynucleotides (CpG-ODN) (10 μg/ml) was added to the appropriate wells. After a further 2 h (IL-6), 4 h (IL-10 and TNFα) or 24 h (IL-12) incubation supernatants were collected and cytokine concentrations determined by ELISA. Results are means ± SD of triplicate assays from a representative experiment;

Fig. 15 are graphs showing cytokine production by antigen- stimulated lymph nodes cells and antigen-specific antibody responses in mice immunised with PBS, 5 μg KLH, 1 μg NP-CyaA + 5 μg KLH or 1 μg P-CyaA + 5 μg KLH. KLH-specific serum antibody titres were measured by ELISA 7 days after immunization (A). Popliteal lymph nodes were harvested and KLH-specific proliferation and cytokine release were assayed (B). Results are mean (± SD) for 5 mice per group and are representative of 3 experiments;

Fig. 16 is a graph showing the effect of immunization with myelin oligodendrocyte (MOG) peptides with CyaA on the disease progression in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis. Mice were immunized subcutaneously (s.c.) with 50 μg MOG peptide (residues 35-55) and 1.0 μg CyaA in phosphate buffered

saline. This was repeated 21 days later. Control mice received MOG peptide or saline only. 7 days after the second immunization, EAE was induced by s.c. administration of 150 μg MOG peptide emulsified in complete Freund's adjuvant, supplemented with 1 mg *Mycobacteria tuberculosis* intraperitoneal (i.p.) injection of 500 ng pertussis toxin, followed 2 days later by a second i.p. injection with 500 ng pertussis toxin. Mice were assessed daily for clinical signs of EAE, and scored as follows: 1 = tail paralysis, 2 = wobbly gait, 3 = hind limb weakness, 4 = hind limb paralysis, 5 = complete paralysis of hind and fore limbs, 6 = death. The disease index was calculated by adding all daily average disease scores, dividing the average day of onset, an multiplying by 100; and

Fig. 17 is a graph showing the effect of immunization with myelin oligodendrocyte (MOG) peptides with CyaA on EAE average disease score over time.

Fig. 18 is a graph showing histopathology section of spinal cords of mice after induction of EAE (untreated) or after immunization with myelin oligodendrocyte peptide (MOG) or MOG peptide + CyaA (MOG + CyaA). EAE was induced and mice immunized as described in Fig. 16, sections of spinal cord were removed from mice 19-23 days after induction of EAE and stained with haematoylin and eosin. The EAE induction in un-treated and MOG-immunized mice is severe with a pronounced mononuclear cell infiltrate; this is considerable reduced in mice immunized with MOG and CyaA.

Detailed description

We have found that adenylate cyclase toxin (CyaA) from *Bordetella pertussis* in combination with a Toll-like receptor (TLR) ligand promotes the induction of interleukin (IL)-10 and IL-6 production by cells of the innate immune system.

Furthermore we have found that CyaA promotes the induction of regulatory T (Tr) cells to co-administered antigens. The induction of Tr cells *in vivo* has potential for the treatment of inflammatory and autoimmune disease and allergy.

We have also found that acylation of the toxin is necessary for cytotoxicity, but not for immunomodulation. A non-palmitoylated CyaA molecule had reduced cyotoxicity, but retained its immunomodulatory function. Therefore *Bordetella pertussis* adenylate cyclase toxin or derivatives thereof have valuable potential as adjuvants, immunotherapeutics or anti-inflammatory agents.

CyaA can subvert host immune responses and thereby contribute to colonisation and persistence of *B. pertussis* in the respiratory tract. Previous investigations have demonstrated adjuvant activity for the wildtype toxin, but the interpretation of these findings was complicated by the presence of lipopolysaccharide (LPS), known to be closely associated with purified CyaA. We examined the adjuvant and immunomodulatory properties of CyaA and the possible contribution of LPS, known to be present in purified CyaA preparations.

CyaA enhanced IL-5 and IL-10 production and IgG1 antibodies to co-administered antigen *in vivo*. Antigen-specific $CD4^+$ T cell clones generated from immunised mice had cytokine profiles characteristic of Th2 and type 1 Tr (Tr1) cells. Since innate immune cells direct the induction of T cell subtypes, we examined the influence of CyaA on activation of dendritic cells (DC) and macrophages. CyaA significantly augmented LPS-induced IL-6 and IL-10 and inhibited LPS driven TNF-$\alpha$ and IL-12p70 production from bone marrow-derived DC and macrophages. CyaA also enhanced cell surface expression of CD80, CD86 and MHC class II on immature DC. The stimulatory activity of the CyaA preparation for IL-10 production and CD80, CD86 and MHC class II expression was attenuated following addition of polymyxin B or with DC from TLR 4-defective mice. Treatment of DC with LPS alone at a concentration present in the CyaA preparation (0.2 ng/ml) failed to

activate DC *in vitro*. We have found that activation of innate cells in vitro by CyaA is dependant on a second signal through TLR4 and that CyaA can promote Th2/Tr1 cell responses by inhibiting IL-12 and promoting IL-10 production by DC and macrophages.

As CyaA causes cytotoxicity of mammalian cells, it may not be suitable for clinical use in humans. However we have prepared non-toxic derivatives and mutants that retain immunomodulatory activity.

CyaA belongs to the RTX family of toxins, which require post translational acylation for activation. Palmitoylation of CyaA is necessary for the toxin to lyse macrophages. Palymitolyated (P-CyaA) and non-palmitoylated CyaA (NP-CyaA) molecules were expressed in *E. Coli* and examined for cytotoxicity and immunomodulatory function. Both proteins enhanced LPS driven IL-10 and IL-6 production and inhibited LPS-stimulated IL-12, TNFá and MIP-1á production in macrophages and DC. At low doses acylated CyaA is more efficient than nonacylated CyaA at eliciting these effects. Both NP-CyaA and P-CyaA also modulated CpG (TLR-9 ligand) and poly(I:C) (TLR3 ligand) as well as LPS-driven cytokine production in DC and macrophages. In addition, despite the lack of acylation CyaA stimulated cAMP accumulation in macrophages and DC. Both proteins stimulated DC maturation leading to increased surface expression of CD80 and MHC-II and decreased expression of LPS-stimulated CD86, CD40 and ICAM-1. The non-acylated CyaA was unable to lyse macrophages or red blood cells even at doses 10 fold higher than that which elicited immunomodulation. Both proteins have similar adjuvant activity *in vivo*, inducing IgG1 antibody and Th2 and Tr cells specific for co-administered antigens. These results indicate that the recombinant non-acylated CyaA molecule lacks cytotoxicity but retains its immunomodulatory effects.

These molecules may be used as adjuvants and therapeutics for the treatment of inflammation or immune mediated diseases. Inflammation and hyperactive T cell responses are features of a number of immune mediated diseases. Autoimmune diseases including multiple sclerosis, rheumatoid arthritis, type 1 diabetes and Crohn's disease involve T cells that secrete interferon (IFN)-$\gamma$, termed type 1 T helper (Th1) cells, and inflammatory responses against self-antigens. In contrast atopic diseases and asthma are mediated by the reciprocal Th2 subtype of T cells.

Many of the diseases detailed above have no satisfactory treatment. Traditional therapies for inflammatory and immune-mediated diseases have largely relied on steroids and non-steroidal anti-inflammatory drugs, however, these are non-specific and have side effects. More recently drugs that inhibit key inflammatory cytokines, in particular tumour necrosis factor (TNF)-$\alpha$, have been developed. These include antibodies or soluble TNF receptors that are effective against certain autoimmune diseases, but are associated with side effects (including recurrent tuberculosis) and are limited to diseases where TNF-$\alpha$ is the key mediator of pathology. Another therapeutic approach is the direct administration of anti-inflammatory cytokines (e.g. IL-10), but this is compromised by the short half-life of the cytokines *in vivo*.

An alternative strategy is to employ agents that induce anti-inflammatory cytokines, such as IL-10, which will have a direct immunosuppressive effect *in vivo* and will also, in the presence of antigen, prime IL-10 secreting antigen-specific Tr cells, which will amplify IL-10 production and the immunosuppressive effect.

We have found that CyaA and derivatives thereof have the potential to drive innate and adaptive IL-10 and thereby act as anti-inflammatory agents and either as immunotherapeutics or as components of vaccines to prevent immune mediated disease. We have shown that CyaA can reduce the severity of disease in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS). Immunization of mice with MOG peptide in the presence of CyaA

delayed the development of EAE and reduced the incidence of disease in EAE, a murine model for multiple sclerosis. CyaA and the non-toxic NP-CyaA have considerable potential as anti-inflammatory agents, immunotherapeutics and adjuvants for vaccines in the prevention of inflammatory or autoimmune diseases.

The invention will be more clearly understood from the following examples thereof.

Examples

**Plasmid construction.** Genomic DNA of *B. pertussis* (strain W28) was prepared from a mid-log culture. The 5' end of *cyaA* was amplified by PCR with oligonucleotides (MWG Biotech, Germany) PAB5 5'-CGCCGGTACCATGCAGCAATCGCATCAGGCT-3' and PAB6 5'-TGGTGAATTCGCTCTTGCCCG-3'. The resulting product was digested with *Kpn*I and *Eco*RI (Invitrogen, CA, USA), inserted in corresponding sites of the cloning vector pBluescript SK- (Stratagene, CA, USA), and this plasmid was named pAPB4. The 3' end of *cyaA* was amplified by PCR from *B. pertussis* genomic DNA with oligonucleotides PAB7 5'-AAGAGCGAATTCACCACATTCGTCG-3' and PAB2 5'-CGCGGATCCTCAGCGCCAGTTGACAGCCA-3'. The product was digested with *Eco*RI and *Bam*HI and ligated into pBluescript SK- at the same restriction sites. This plasmid was named pAPB5, and was then digested with *Eco*RI and *Bam*HI and the 3' *cyaA* fragment was subcloned into the corresponding sites of pAPB4 giving a full-length *cyaA* gene. This plasmid was named pAPB6. *cyaC* was amplified by PCR from the genomic DNA of *B. pertussis* with oligonucleotides PAB3 5'-CGCGGATCCGAGGGCATGTCATGCTTCCGTCCGCC-3' and PAB4 5'-CGCGGCGAAGCTTTCAGGCGGTGCCCCGGC-3'. The PCR fragment was digested with *Bam*HI and *Hin*DIII and cloned into the pASK-IAB6 (IBA GmbH, Germany) expression vector opened with the same restriction enzymes. This new plasmid was termed pAPB1. The intact *cyaA* gene was isolated from pAPB6 digested with *Kpn*I and *Bam*HI, cloned into pAPB1 upstream of the *cyaC* gene using the *Kpn*I and *Bam*HI sites, and this plasmid was termed pAPB8. pAPB8 was

digested with *Kpn*I and *Hin*DIII and the 5.9 kb product containing *cyaA* and *cyaC* was cloned into the commercial His-tagged vector pQE-80 (Qiagen, UK) opened at the same restriction sites. The sequence and orientation of cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech). This plasmid was named pJR2 from which His-tagged palmitoylated CyaA could be expressed in *E. coli*.

**Purification of CyaA.** *E. coli* XL-1 Blue (pJR2) was induced to express CyaA and CyaC by the addition of isopropyl-β-thiogalactopyranoside (IPTG, Bioline, UK) to an exponentially growing bacterial culture in Luria-Bertani (LB) broth supplemented with 150 μg/ml ampicillin with vigourous shaking at 37°C. The bacterial culture was centrifuged and the bacterial pellet resuspended in 50 mM Tris-HCl, 0.2 mM $CaCl_2$, pH 8.0, supplemented with protease inhibitor cocktail (P-8465 Sigma, UK). Bacteria were disrupted with FastPrep Protein Blue beads (QbioGene, CA, USA) in a FastPrep machine at speed 6 for 20 sec. The insoluble material containing CyaA was separated by centrifugation, washed with 50 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2% Triton X-100, pH 8.0 and incubated in 50 mM Tris-HCl, 0.2 mM $CaCl_2$, 8 M urea, pH 8.0 (Buffer A) for 1 h at room temperature with stirring. The solubilised CyaA was collected following centrifugation. After addition of NaCl to a final concentration of 0.1 M, CyaA was loaded on a DEAE cellulose (Sigma) column equilibrated with Buffer A supplemented with 0.1 M NaCl and eluted with Buffer A supplemented with 0.2 M NaCl as previously described (48). The protein was further purified on $Ni^{++}$ columns (Qiagen) under denaturing conditions by pH adjustment as recommended by the manufacturers and eluted in 100 mM $NaHPO_4$, 10 mM Tris-HCl, 8 M urea, 0.2 mM $CaCl_2$, pH 4.5. LPS removal was attempted using Detoxigel endotoxin removal columns (polymyxin B conjugated columns, Pierce, IL, USA) following the manufacturer's protocols. LPS was dissociated from CyaA by dialysis first against Dulbecco's PBS (Sigma), 1 mM EDTA, 1 M urea, pH 4.6 and then against Dulbecco's PBS, 0.1 mM $CaCl_2$, 2 M urea pH 8.0. The purified protein was stored in aliquots at -20□C. LPS was measured by a colorimetric limulus amaeobocyte lysate assay (QCL-1000; Biowhittaker, MD, USA) and protein

concentrations were determined by Bradford (Bio-Rad). Proteins were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised with Coomassie Blue (GelCode Blue Stain Reagent, Pierce). Alternatively proteins were transferred to a nitrocellulose membrane following SDS-PAGE and probed with anti-His Tag antibodies (Santa Cruz Biotechnologies) and anti-CyaA antibodies (kind gift from Erik Hewlett). The bands were visualised by incubation with secondary anti-rabbit IgG horseradish peroxidase conjugated antibodies (Sigma) and chemiluminescent supersignal detection system (Pierce).

**Cloning, expression and purification of CyaA.** *cyaA*, the gene encoding CyaA, and *cyaC*, the gene whose product is required to post-translationally activate CyaA, were cloned from the genomic DNA of *B. pertussis* W28 into pQE-80 to allow inducible expression of these genes in *E. coli*. This plasmid pJR2, expressing 6xHis-tagged CyaA, was introduced into electrocompetent *E. coli* XL1-blue cells. Bacteria harbouring the recombinant plasmid were recovered and the correct orientation and position of the cloned genes were confirmed by both restriction digestion and sequencing.

Active CyaA was solubilised from bacterial inclusion bodies and purified on DEAE and $Ni^{++}$ columns. The purity of the 200 kDa CyaA protein was greater than 95% as estimated by Coomassie staining following gel electrophoresis and this band was recognised by both anti-His and anti-CyaA antibodies (data not shown). The LPS content of the CyaA protein was monitored throughout the purification process. At this stage the protein preparation contained significant amounts of LPS (>2 ng LPS/μg protein). A number of procedures were used to attempt to remove the LPS. Passage of the protein preparation through polymyxin B conjugated columns reduced, but did not eliminate, the LPS. This suggested that CyaA was closely complexed with LPS, as has been previously reported for other RTX toxins. LPS can be dissociated from proteins by the addition of EDTA to chelate calcium ions and by lowering the pH to below the pI of the protein. After dialysis of the protein in these conditions, the preparations of CyaA contained 100-250 pg LPS /μg protein.

This concentration of LPS did not stimulate macrophages or DC *in vitro* (data not shown; and also Fig. 4, 5 and 7). This preparation was used in the adjuvant and immunomodulatory studies described below.

**In vitro CyaA enzymatic activity.** 0.1 μg protein was incubated for 5 min at 30°C in 50 μl 50 mM Tris-HCl, 100 μg/ml BSA, 0.1 μM calmodulin, 0.12 mM $CaCl_2$, 6 mM $MgCl_2$, 2 mM ATP, pH 8.0. The reaction was mixed with lysis reagent 1B of the Amersham Biosciences Biotrak enzymeimmunoassay system and boiled for 5 min. cAMP was measured by a competitive ELISA in the Amersham kit. Units are μmol cAMP produced per min at 30°C, pH 8.0.

**Intracellular cAMP accumulation.** Macrophages (J774 cell line) and DC were cultured at $1x10^6$ cells/ml in DMEM or RPMI medium respectively with CyaA (1 μg/ml). After 30 min, 4 h and 24 h incubation, lysis reagent 1B of the Amersham Biosciences Biotrak Enzymeimmunoassay system was added and the samples processed for quantification by cAMP ELISA.

**LDH assay.** The lysis of J774 macrophages was measured by the release of lactose dehydrogenase (LDH) into the culture supernatants using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, WI, USA). Cells ($1x10^5$ /100 μl) were aliquoted into a 96-well plate in DMEM. CyaA was added at the specified concentrations and the plates incubated at 37□C with 5% $CO_2$ for the indicated amounts of time. 100% lysis wells were prepared by the addition of lysis reagent to the appropriate wells. Supernatants (50 μl) were removed from each well for the LDH assay. Percentage lysis = [(OD of sample - OD of untreated cells)/(OD of 100% lysis cells - OD of untreated cells)]*100.

**CyaA biochemical properties.** The CyaA preparation was analysed biochemically to ensure that both its enzymatic and membrane translocation properties were active. *In vitro* assays of the adenylate cyclase enzyme function showed that the protein was

enzymatically active (26.6 ± 0.8 units/mg). CyaA at a concentration of 1 μg/ml was able to increase the intracellular concentration of cAMP 80-fold in J774 macrophages (72.8 ± 2.9 vs 0.9 ± 0.1 pmol cAMP/$10^6$ cells), showing that the protein has the ability to target and enter eukaryotic cells. The maximal cAMP concentration was reached within 30 min and was maintained for at least 24 h. This concentration of CyaA induced less than 1% cell lysis after 24 h, as measured by LDH release, however lysis (up to 10%) was observed with CyaA at concentrations of 5–10 μg/ml (data not shown). Therefore to assess its immunomodulatory function CyaA was used at 1 μg/ml, a concentration that induces a large increase in intracellular cAMP, without affecting cell viability.

**Animals and immunisation.** Female specific pathogen free BALB/c, C3H/HeN and C3H/HeJ mice were purchased from Harlan Olac (Bicester, UK) and used at 6-8 wk old, with 4 or 5 mice per group. Mice were housed in individually ventilated cages and all experiments were performed according to regulations of the Irish Department of Health, the EU and the Ethics Committee of Trinity College Dublin. Mice were immunized subcutaneously (s.c.) in the hind footpads once or twice (0 and 21 days) with depyrogenated keyhole limpet hemocyanin (KLH; 5 μg; Calbiochem, La Jolla, CA, USA), KLH (5 μg) with CyaA (1 μg) or with Dulbeccos PBS (Sigma, Poole, UK) in a final volume of 50 μl. Seven days after the first or second immunisation mice were sacrificed by cervical dislocation and serum and popliteal lymph nodes collected.

**Generation of Ag-specific T cell lines and clones.** Popliteal lymph node cells (1 x $10^6$/ml) from immunized mice were cultured with KLH (50 μg/ml). After two round of antigen stimulation, T cell lines were cloned by limiting dilution. T cell lines and clones were maintained by culture with antigen (KLH, 50 μg/ml) and splenic APC for 4-5 days, followed by 5-7 days culture with irradiated feeder cells and IL-2. T cells were tested for cytokine production at the end of the starve cycle.

**Antigen-specific cytokine production.** Lymph node cells (1 x $10^6$ cells/ml) from immunized mice or T cell lines or clones and APC (irradiated spleen cells, 2 x $10^6$/ml) were cultured at 37ºC and 5% $CO_2$ in RPMI medium with KLH (2-50 μg/ml) or phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and anti-CD3 (0.5μg/ml; BD, Pharmingen) or medium only. After three days, supernatants were collected for cytokine detection and the medium replaced. On the following day $^3$H-Thymidine (950 μCi/well; Amersham Pharmacia, UK) was added and the cells cultured for a further 5 h, after which cells were harvested and proliferation assessed by $^3$H-Thymadine incorporation. Concentrations of IL-4, IL-5 and IFN-γ were determined by immunoassay using pairs of antibodies and recombinant cytokines (BD Pharmingen, San Diego, CA) as standards. IL-10 concentrations were determined using a commercially available Duo-Set kit kits (R&D Systems, Minneapolis, U.S.A.)

**Antibody assays.** Titres of KLH-specific IgG, IgG1 and IgG2a in the serum of immunized mice were determined by ELISA.

**CyaA generates Th2 and Tr1 cells to co-injected antigen.** To examine the adjuvant properties of CyaA, mice were immunized s.c. in the hind footpad with KLH (5 μg), alone or with CyaA (1 μg). Seven days later mice were sacrificed and lymph node cells were re-stimulated with antigen (KLH 2-50 μg/ml) *in vitro*. Cytokine concentrations were determined in supernatants removed after 3 days and proliferation was assessed after 4 days. Immunization with KLH alone induced weak cellular immune response; only IL-4 production was enhanced over that observed in mice immunized with PBS (Fig. 1). In contrast, significant antigen-specific proliferation was observed in cells from the re-stimulated lymph node cells from mice immunized with KLH and CyaA. Furthermore, significantly higher concentrations of KLH-specific IL-10 and IL-5 were detected in lymph node cells from mice immunized with CyaA and KLH compared to mice immunized with KLH alone. IL-4 and IFN-γ were also enhanced, but the difference between mice that received KLH alone and KLH and CyaA was, in most cases, not significant. A

similar pattern of cytokine secretion was observed 7 days after a booster immunization (data not shown).

The cytokine profile of antigen-stimulated lymph node cells indicates that CyaA enhances Th2 and /or Tr1 cells to co-administered antigens. To confirm this KLH-specific $CD4^+$ T cell lines and clones from mice immunized with KLH in the presence of CyaA were generated. Each of the 10 T cell lines examined secreted high levels of IL-10 and lower levels of IL-5 and a smaller number also secreted IL-4 (Fig. 2A). IFN-γ production was detectable in 6 of 10 T cell lines examined and at high levels in only one of these T cell lines (Fig. 2A). In contrast, IFN-γ was produced at a concentration in excess of 50 ng/ml by all T cell lines from mice immunized with KLH in the presence of CpG-oligodeoxynucleotides (not shown). A number of the T cell lines generated from mice immunized with KLH and CyaA were cloned and cytokine-production by T cell clones from one representative T cell line is shown in Fig. 2B. These KLH-specific T cell clones secreted IL-5 and IL-10, or IL-4, IL-5 and IL-10 but undetectable IFN-γ, profiles characteristic of Tr1 and Th2 cells respectively. This indicates that CyaA promotes the induction of Th2 and Tr1-type cells specific for the co-administered antigen.

**CyaA enhances IgG1 responses to co-administered antigen.** We examined the adjuvant effect of CyaA for antibody responses to co-injected antigen by assessing KLH-specific IgG and IgG subclasses in mice immunized with KLH alone or with CyaA. Significantly higher levels of KLH-specific IgG1 were found in the serum of mice immunized with KLH and CyaA compared with mice that received antigen alone. In contrast, CyaA did not enhance IgG2a levels above those observed in mice immunized with KLH alone (Fig. 3A). Following a second immunization the serum IgG titres were increased over those observed after a single immunization and the responses to KLH in the presence of CyaA were significantly greater than those in mice immunized with KLH alone (Fig. 3B). Comparable with the data after a single immunization, IgG1 was the dominant subclass of the antibody response. This

clearly demonstrates that CyaA acts an adjuvant for antibody, as well as T cell responses *in vivo*.

**Effect of CyaA on cytokine and chemokine release by J774 macrophages and murine bone marrow derived dendritic cells.** Murine bone marrow derived dendritic cells (BMDC) were prepared from bone marrow from the femur and tibia of mice, which was cultured for 3 days in RPMI medium supplemented with 10% supernatant from a GM-CSF secreting cell line, J558-GM-CSF. Non-adherent cells were recovered and resuspended in fresh media with 10% GM-CSF cell supernatant. On day 7, cells were collected, washed and recultured in RPMI medium and used for immunoassays. J774 macrophages were cultured in DMEM supplemented with 8% FCS at 37°C with 5% $CO_2$ and used before the twentieth passage. Macrophages and DC ($1x10^6$ cells/ml) were cultured with CyaA (1 μg/ml) alone or 2 h later with LPS (Sigma, 1-1000 ng/ml). Polymyxin-B (10 μg/ml) was added where indicated. Supernatants were collected after 2, 4 and 28 h for analysis of cytokine production and cell surface marker expression. Concentrations of IL-10, IL-12p70, and TNF-α in cell supernatants were determined using commercially available Duo-Set kits (R&D Systems). Concentrations of IL-6 and macrophage inflammatory protein (MIP)-1α were determined by immunoassay using pairs of antibodies and recombinant cytokines as standards (BD Pharmingen).

**CyaA modulates cytokine production from innate cells.** Cells of the innate immune system, including DC and macrophages direct the adaptive immune response by presenting antigens and secreting regulatory cytokines. To investigate the effect of CyaA on these cells, J774 macrophages and immature bone marrow-derived DC were incubated with CyaA (1 μg/ml), LPS (1-1000 ng/ml) or CyaA and LPS. Since the CyaA protein is associated with LPS, which was reduced but not completely eliminated during purification, it was important to determine the role, if any, of this LPS in the immunomodulatory effects of CyaA. Therefore cells were also stimulated with CyaA in the presence of polymyxin B. Supernatants were

collected 2, 4 and 28 h after stimulation and assayed for cytokines. The purified CyaA, that included residual LPS (220 pg/ml), stimulated low levels of IL-6, IL-10 and TNF-$\alpha$ production from J774 cells (Fig. 4) and low levels of IL-6 and TNF-$\alpha$ (but no IL-10) secretion from DC (Fig. 5). This cytokine production was abrogated in the presence of polymyxin B. However, stimulation with LPS alone at the dose present in the CyaA preparation (220 pg/ml) did not induce production of these cytokines (Figs 4 and 5). This indicates that CyaA activates innate cells only in the presence of LPS. Therefore we examined the effect of CyaA on cytokine production in response to increasing doses of LPS. CyaA synergised with LPS in promoting IL-6 and IL-10 production from macrophages and DC. IL-10 production from macrophages stimulated with CyaA and LPS (1-1000ng/ml) was significantly higher than that of macrophages stimulated with the corresponding dose of LPS alone at all time points examined (Fig. 4). IL-10 could not be detected in DC supernatants 4 h after stimulation with LPS (1-1000 ng/ml) alone, whereas significant levels of IL-10 were produced following addition of CyaA (Fig. 5). LPS-induced IL-6 production by macrophages and DC was also significantly enhanced by the addition of CyaA, but this was only observed at early time points. In contrast to the positive effect on IL-6 and IL-10 production, CyaA suppressed TNF-$\alpha$ secretion from macrophages and DC and IL-12p70 production from DC. These inhibitory effects were observed at the three time points examined and over a range of doses of LPS. This demonstrates that CyaA alone has little enhancing effect on cytokine production by cells of the innate immune system, but can synergise with LPS, even at very low concentrations, in promoting IL-6 and IL-10 production, but also inhibiting TNF-$\alpha$ and IL-12 production. Similar results were observed when TLR9 and TLR3 ligands, CPG-ODN or poly(I:C), were used in place of LPS (data not shown).

**Analysis of DC maturation.** DC ($1x10^6$/ml) were cultured for 24 h with CyaA (1 μg/ml), *E.coli* LPS (10 ng/ml or 1 μg/ml) or CyaA in the presence of polymyxin-B (10 μg/ml; Sigma) as indicated. Cells were recovered and surface marker expression assessed by flow cytometry using fluorescently labelled antibodies (BD

Pharmingen). Cells were incubated for 30 min at 4°C with antibodies specific for mouse CD80 (Hamster IgG2, clone 16-10A1), CD86 (Rat IgG2a, clone GL1), CD11c (Hamster IgG1, clone HL3), MHC class II (mouse IgG2b, I-$A^d$, clone AMS-32.1), CD40 (Rat IgG2a, clone 3/23) or ICAM-I (Hamster IgG1, clone 3E2), followed by washing and incubation with streptavidin-PerCP in the case of biotin labelled primary antibodies. Cells labelled with appropriate isotype matched antibodies with irrelevant specificity acted as controls. 30,000 cells per sample were analysed on a FACScaliber flow cytometer. Analysis was performed on CD11c gated cells using CellQuest V3.3 software (Becton Dickinson Immunocytmetery Systems, San Jose, CA).

**Statistics.** Cytokine and Chemokine levels were compared by one-way analysis of variance (ANOVA). Where significant differences were found, the Tukey Kramer multiple comparisons test was used to identify differences between individual groups.

**Effect of CyaA on maturation of DC.** Several pathogen-derived molecules that bind to TLRs, induce maturation of immature DC, thereby enhancing their capacity to activate naïve T cells. Therefore, we examined the ability of CyaA to stimulate the maturation of DC and/or to modulate LPS-induced maturation. Immature DC were stimulated with CyaA, LPS or LPS with CyaA and the expression of surface markers associated with maturation was examined by immunoflourescence analysis 24 h later. As expected, LPS (1 μg/ml), enhanced surface expression of CD80, CD86, ICAM-I, CD40, and MHC class II (Fig. 6). Stimulation of immature DC with CyaA (in the presence of polymyxin B) also resulted in upregulation of surface expression of CD80 and MHC class II, and to a lesser extent CD86. In contrast, expression of CD40 and I-CAM I was downregulated following incubation with CyaA. Furthermore, CyaA inhibited LPS-induced upregulation of CD40, ICAM-I and CD86. In contrast, treatment of cells with LPS at the level present in the CyaA preparation (220 pg/ml) had no effect on DC surface marker expression (data not shown). The results indicate that CyaA treatment results in partial maturation of the

DC, upregulating CD80 and MHC-II, but inhibits CD40 and ICAM-1, a phenotype distinct from mature DC that drive the differentiation of Th1 cells, but similar to those that promote the induction of Tr1 cells.

**Effect of CyaA on DC from TLR-4 defective mice.** To further address the role of LPS in the mechanism of action of CyaA as an adjuvant and immunomodulator, we examined innate cytokine and chemokine production and maturation of DC from TLR4-defective mice. DC from C3H/HeN and C3H/HeJ mice were treated with CyaA, LPS, LPS and CyaA or CyaA and polymyxin B and supernatants recovered after 4 h. CyaA (that included 220 pg/ ml residual LPS) stimulated IL-10 production by DC from C3H/HeN, but not from C3H/HeJ mice (Fig. 7). Furthermore, IL-10 production by DC from C3H/HeN mice was inhibited by polymyxin B. However, LPS alone at the concentration present in the CyaA preparation (220 pg/μg) did not induce IL-10, IL-12p70, TNF-$\alpha$ or MIP-1$\alpha$ production by DC from C3H/HeN mice. Addition of a higher dose of exogenous LPS (10 ng/ml) did not induce IL-10 at the 4 h time point, but synergized with CyaA in promoting IL-10 production. Furthermore, CyaA suppressed LPS induced IL-12p70, TNF-$\alpha$ and MIP1-$\alpha$ production by DC from C3H/HeN mice (Fig. 7), but had no effect on cytokine production by DC from C3H/HeJ mice (Fig. 7). In contrast, CpG, a TLR9 ligand, activated cytokine production by C3H/HeN and C3H/HeJ DC in a similar fashion (Fig. 7). The results indicate that CyaA, although having no direct effect on cytokine production by DC, modulates LPS-induced responses, synergising with LPS to induce IL-10 and suppressing LPS induced production of IL-12p70, TNF-$\alpha$ and MIP-1$\alpha$.

The effects of CyaA on maturation of DC from C3H/HeN and C3H/HeJ mice were also examined. As shown for BALB/c mice, CyaA induced maturation of DC from C3H/HeN mice, specifically CyaA enhanced expression of CD80, CD86, MHC class II, CD40 and ICAM-1 (Fig 8A). In the presence of polymyxin B these effects were diminished, in particular CD40, ICAM-1, and MHC class II, which were expressed at lower levels than those seen on medium-treated control DC. LPS-induced

expression of CD86, CD40 and ICAM-1 were also inhibited by CyaA, though not to the same extent as that observed in DC from BALB/c mice. In contrast to the modulatory effects in C3H/HeN mice, LPS, CyaA or LPS with CyaA had no enhancing effects on CD86, MHC class II, CD40 or ICAM-1 and had a modest effect on CD80 on DC from C3H/HeJ mice (Fig. 8B). However CyaA marginally reduced CD40 and ICAM-1 expression on DC from C3H/HeJ mice in the presence of polymyxin B. The results indicate that upregulation of maturation markers on DC by CyaA is dependant on LPS even at very low doses, but that inhibition of endogenous expression of CD40 and ICAM-1 can occur in the absence of LPS.

**Haemolysis assay** - Defibrinated sheep blood (Cruinn) was diluted 1:1 in TNC buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 8.0), layered on Ficoll Histoplaque and centrifuged at 1500 rpm for 30 min. The pellet of red blood cells was washed twice in TNC buffer and diluted to 5 x $10^8$ cells/ml. 100 µl cells was aliquoted in 96-well plate and CyaA added at the concentrations indicated in the text. The plates were incubated at 37°C for 18h. As positive control 10 µl 10% SDS was added to wells containing untreated cells. The plates were centrifuged at 2200 rpm for 15 min at 4ºC. The supernatants were transferred to a fresh 96 well plate and measured at 541 nm for the release of haemoglobin. Percentage lysis = [(OD of sample - OD of untreated cells)/(OD of 100% lysis cells - OD of untreated cells)]*100.

**Purification and biochemical characterisation of palmitoylated and non-palmitoylated CyaA** -Recombinant His-tagged fusion proteins of palmitoylated and non-palmitoylated CyaA were purified from *E. coli* as previously described. Palmitoylated CyaA (P-CyaA) was purified from *E. coli* XL-1 Blue (pJR2) expressing His-CyaA and CyaC together under the control of the IPTG-inducible promoter $p_{tac}$. Non-palmitoylated CyaA (NP-CyaA) was purified from *E. coli* XL-1 Blue (pJR1), a similar plasmid that lacks *cyaC*. The LPS content of each final protein preparation was 184 pg LPS/µg NP-CyaA and 191 pg LPS/µg P-CyaA. Both

proteins were enzymatically active with specific activities for NP-CyaA and P-CyaA of 30.6 and 39.6 µmol cAMP generated/min/mg respectively. So the purified NP-CyaA and P-CyaA were judged to be biochemically equivalent as similar results were obtained for each protein in these in vitro assays.

**Palmitoylation of CyaA is required for lysis of RBC and macrophages-** Palmitoylation had previously been shown to be necessary for CyaA to bind to red blood cells, increase intracellular cAMP in these cells and cause haemolysis (1). In addition, non-palmitoylated CyaA was unable to lyse J774 macrophages or Jurkat T-cells (1, 13), though whether NP-CyaA binds to or causes cAMP accumulation in these cells was not reported. Recently it has been shown that NP-CyaA is unable to lyse or to cause cAMP accumulation in CHO cells transfected with CD11b, but it can bind these cells very efficiently in a CD11b dependant manner, though not as tightly as P-CyaA (14). Other RTX proteins require modification in order to lyse target cells, but whether modification is necessary for cell binding is unknown. We examined the ability of NP-CyaA and P-CyaA to lyse red blood cells and J774 macrophages. P-CyaA lysed 8.9 ± 4.0% red blood cells and 10.9 ± 3.6% macrophages at a concentration of 10 µg/ml, however the same concentrations of NP-CyaA only caused minimal lysis (0.3 ± 0.6% for red blood cells, 1.0 ± 1.0% for macrophages) (Fig. 9). No lysis was detected at lower doses of P-CyaA or NP-CyaA. Thus, palmitoylation is required for CyaA to lyse cells, as is the case for other RTX toxins.

**Palmitoylation is not required for the cAMP driven modulation of cytokine release by CyaA treated macrophages.** The influence of palmitoylation on the immunomodulatry activity of CyaA on innate immune cells was examined. J774 macrophages were treated with 1 µg/ml of each CyaA alone or in the presence of 10 µg/ml polymyxin B, to negate any effects of the low concentrations of remaining LPS. LPS (1 µg/ml) was added to the indicated wells 2 hours later and after a further 4 h incubation the concentrations of intracellular cAMP and of the secreted

chemokines and cytokines were quantified. Surprisingly, NP-CyaA and P-CyaA caused similar increases in cAMP accumulation in macrophages (1.48 and 1.51 nmol cAMP/$10^6$ cells, respectively). The cAMP concentration in untreated cells was 0.001 nmol cAMP/$10^6$ cells. cAMP concentrations in CyaA-treated or untreated cells were unaffected by polymyxin B or by LPS (data not shown). Furthermore, NP-CyaA synergised with LPS to drive IL-10 secretion in a manner similar to P-CyaA though to a lesser extent (Fig. 10). NP-CyaA was as effective as P-CyaA at inhibiting LPS-driven TNF$\alpha$ secretion and also reduced MIP-1á production in response to LPS, though to a lesser extent than P-CyaA. Neither NP-CyaA nor P-CyaA alone or in combination with polymyxin B induced IL-10, TNF$\alpha$ or MIP-1$\alpha$ secretion in macrophages significantly above background. IL-12 p70 was not detected in any of the samples (data not shown). These data indicate that NP-CyaA was capable of modulating LPS-driven responses in macrophages but less efficiently than P-CyaA. Therefore we tested the responses of these cells to a range of doses of the proteins (0.1 μg/ml to 3 μg/ml) in the presence or absence of LPS. The data show that at doses of 1 μg/ml and above NP-CyaA and P-CyaA similarly affect cytokine responses in these cells, while at lower doses NP-CyaA shows significantly less modulatory activity than P-CyaA (Fig. 11). The results indicate that palmitoylation is not an absolute requirement for CyaA to cause an increase in intracellular cAMP concentrations or to modulate macrophage cytokine or chemokine release, but it does increase the efficiency of these effects.

**Palmitoylation is not required for the modulation of DC activation by CyaA -** BMDC were generated from C3H/HeN and from BALB/c mice. DCs from both strains of mice responded similarly (Fig. 11 and data not shown). NP-CyaA and P-CyaA synergised equally efficiently with LPS to enhance IL-10 secretion by DC (Fig. 12). LPS stimulation of TNFá, IL-12p70 and MIP1$\alpha$ secretion was suppressed by both CyaA proteins. Although the decrease in the release of LPS-stimulated TNFá was less for NP-CyaA when compared with P-CyaA, the reduction was still significant. Treatment of DC with NP-CyaA alone induced the secretion of IL-10

and TNFα, while treatment with P-CyaA alone induced IL-10. We have previously shown that P-CyaA synergises with LPS in the protein preparations to stimulate cytokine production from DC. The addition of polymyxin B abrogated the production of these cytokines indicating that these cytokines were secreted as a result of the synergy between LPS and P-CyaA or NP-CyaA. This was further substantiated by the use of DC derived from C3H/HeJ mice that have a point mutation in the cell surface LPS receptor TLR4 that renders these mice hyporesponsive to LPS. Neither NP-CyaA nor P-CyaA induced cytokine secretion from these DC, indicating that the increase in TNFα is due to the LPS in these protein preparations and that this LPS is synergising with each of these proteins to drive IL-10 production.

Maturation of DC in response to pathogen-derived molecules is characterized by an increase in cell surface expression of MH class II and co-stimulatory molecules, such as CD80, Cd86 and CD40. We have previously shown that P-CyaA can influence the cell surface marker expression of unstimulated DC and of DC treated with LPS. Here we examined the influence of NP-CyaA on DC from BALB/c mice. Both NP-CyaA and P-CyaA enhanced surface expression of CD80 and MHC-II and suppressed LPS-induced surface expression of CD86, CD40 and ICAM-1 (Fig. 13). The results indicate that palmitoylation is not required for CyaA to modulate DC activation, determined either by chemokine and cytokine release or by cell surface marker expression.

We have shown that CyaA can synergise with LPS to activate certain responses in innate immune cells, possibly as a result of co-operative recognition of a an integrrin (CD11b/Cd18) and a TLR. Analogous to LPS activation of innate immune cells through TLR4, CpG motifs in bacterial DNA can signal through TLR9. We examined whether CyaA can modulate CpG stimulation of cytokine responses from DC. C3H/HeN and C3H/HeJ DC were incubated with P-CyaA or NP-CyaA, CpG-ODN (10 μg/ml) was added after 2h and cytokine levels in the supernatants were

measured 4 h (IL-10 and TNFα) or 24 h (IL-12) later. CpG-stimulated TNFα and IL-12 production by C3H/HeJ DC was down regulated by P-CyaA and NP-CyaA, while IL-10 levels were neglible in both C3H/HeJ and C3H/HeN DC (Fig 14A). In C3H/HeN DC the results were complicated by the synergy of CpG with the LPS in the CyaA protein preparations. Increased levels of TNFα and IL-12 secretion from NP-CyaA plus CpG treated DC as compared to CpG treated cells were observed. P-CyaA did not modify production of these cytokines by CpG-treated cells. To further examine the effects of CyaA on CpG signalling J774 macrophages were incubated with P-CyaA or NP-CyaA and CpG-ODN, in the presence or absence of polymyxin B. Both P-CyaA and NP-CyaA diminished production of TNFá and increase IL-6 and IL-10 production from CpG stimulated macrophages (Fig 14B). These effects were only observed in the cells cultured in the absence of polymyxin B.

**Palmitoylation is not required for the adjuvant activity of CyaA** - As NP-CyaA had similar activity to P-CyaA in modulation macrophage and DC function in vitro, we examined the ability of NP-CyaA and P-CyaA to act as adjuvants in vivo. Mice were immunized s.c in the footpad with KLH alone or with NP-CyaA and P-CyaA. 7 days later popliteal lymph nodes were harvested and antigen-specific T-cell responses were measured. Both proteins gave comparable results. Ex vivo stimulation of popliteal lymph node cells with KLH lead to significantly more proliferation (Fig. 15B) and higher secretion of IL-10, IL-4 and IL-5 by groups treated with KLH plus P-CyaA or NP-CyaA than the group treated with KLH alone. IFN-ã secretion was also detected in comparable amounts. KLH-specific antibody titres in both the CyaA groups were significantly higher than the KLH alone group and IgG1 was the predominant subclass, indicating a Th2 response (Fig. 15A). Thus NP-CyaA has similar adjuvant activity to P-CyaA and retains the ability to enhance Th2 and Tr1 response in vivo.

**Murine model for multiple sclerosis**

Experimental autoimmune encephalomyelitis (EAE) is a murine model for multiple sclerosis. EAE is induced in C57BL/6 mice by s.c. administration of 150 μg MOG peptide emulsified in complete Freund's adjuvant, supplemented with 1 mg *Mycobacteria tuberculosis* intraperitoneal (i.p.) injection of 500 ng pertussis toxin, followed 2 days later by a second i.p. injection with 500 ng pertussis toxin. Mice develop symptoms of paralysis. In experiments to assess the effects of CyaA as an adjuvant for a vaccine against autoimmune disease, mice were immunized subcutaneously (s.c.) with 50 μg MOG peptide (residues 35-55) and 1.0 μg CyaA in phosphate buffered saline. This was repeated 21 days later. Control mice received MOG peptide or saline only. 7 days after the second immunization EAE was induced with MOG, Freund's adjuvant and pertussis toxin as described above. Mice were assessed daily for clinical signs of EAE, and scored as follows: 1 = tail paralysis, 2 = wobbly gait, 3 = hind limb weakness, 4 = hind limb paralysis, 5 = complete paralysis of hind and fore limbs, 6 = death.

Table 1 shows the disease score and disease index results. The results indicate that the administration of CyaA as an adjuvant significantly inhibits disease progression.

Table 1

| Immunization Group | Incidence | Day of onset | Mean Max Clinical Score | Disease Index at day 23 |
|---|---|---|---|---|
| Control | 10/11 | 16.4 | 2.9 | 195 |
| MOG | 7/8 | 15 | 1.875 | 100 |
| MOG + ACT | 8/8 | 17.6 | 1.125 | 6.39 |

Incidence is the number of mice out of the number tested that develop any clinical symptoms of EAE. The disease index was calculated by adding all daily average disease scores, dividing the average day of onset, and multiplying by 100.

The invention is not limited to the embodiments hereinbefore described which may be varied in detail.

References

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Claims

1. A method for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

2. A method for the treatment and/or prophylaxis of an immune-mediated disorder comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

3. A method for the treatment and/or prophylaxis of an autoimmune disease comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

4. A method as claimed in any of claims 1 to 3 wherein the adenylate cyclase toxin (CyaA) is combined with self or foreign antigens or peptides thereof.

5. A method as claimed in any of claims 1 to 4 wherein the adenylate cyclase toxin (CyaA) is derived from *Bordetella pertussis, Bordetella bronchisepetica* or *Bordetella parapertussis*.

6. A method as claimed in any of claims 1 to 5 wherein the agent modulates inflammatory cytokine production.

7. A method as claimed in any preceding claim wherein the immunomodulatory effects of CyaA on cells of the innate immune system is dependent on co-activation with a Toll-like receptor (TLR) ligand.

8. A method as claimed in claim 7 wherein the Toll-like receptor (TLR) ligand is LPS or other toll-like receptor ligands, including CpG motifs, dsRNA, Poly (I:C).

9. A method as claimed in any preceding claim wherein CyaA promotes IL-10 and IL-6 production by macrophages and dendritic cells (DC).

10. A method as claimed in any preceding claim wherein CyaA synergises with LPS to promote IL-10 and IL-6 production by macrophages and dendritic cells (DC).

11. A method as claimed in any preceding claim wherein CyaA inhibits inflammatory cytokines, chemokines or other inflammatory mediators.

12. A method as claimed in claim 11 wherein the inflammatory cytokine is selected from any one or more of IL-12 or TNF-$\alpha$, IFN-$\gamma$, IL-1, IL-23 or IL-27.

13. A method as claimed in claim 11 wherein the inflammatory chemokine is macrophage inflammatory protein-1$\alpha$ or macrophage inflammatory protein-1$\beta$.

14. A method as claimed in any preceding claim wherein CyaA promotes dendritic cell maturation following co-activation with TLR-ligands.

15. A method as claimed in claim 14 wherein CyaA promotes CD80 expression by dendritic cells.

16. A method as claimed in any preceding claim wherein CyaA inhibits TLR-ligand-induced dendritic cell activation.

17. A method as claimed in claim 16 wherein CyaA inhibits CD40 and ICAM-1 expression.

18. A method as claimed in any preceding claim wherein CyaA acts as an adjuvant *in vivo* to promote the induction of Th2 or Tr cells to co-administered antigens.

19. A method as claimed in any preceding claim wherein CyaA acts as an adjuvant *in vivo* to promote IgG1 antibodies to co-administered antigens.

20. A method as claimed in any preceding claim wherein the CyaA is present in a non-palmitoylated form.

21. A method as claimed in any preceding claim wherein the CyaA is in the form of an adjuvant, immunotherapeutic of anti-inflammatory agent.

22. A method as claimed in any preceding claim wherein the agent modulates inflammatory cytokine production induced by infection or trauma.

23. A method as claimed in any preceding claim wherein the disorder is sepsis or acute inflammation induced by infection, trauma or injury.

24. A method as claimed in any preceding claim wherein the disorder is selected from any one or more of Crohn's disease, inflammatory bowel disease, multiple sclerosis, type 1 diabetes or rheumatoid arthritis.

25. A method as claimed in any preceding claim wherein the disorder is asthma or atopic disease.

26. A method as claimed in any preceding claim in wherein the agent is in a form for oral, intranasal, intravenous, intradermal, subcutaneous or intramuscular administration.

27. A pharmaceutical composition comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

28. An immunomodulator comprising adenylate cyclase toxin (CyaA).

29. A recombinant non-acylated CyaA having immunomodulatory effects.

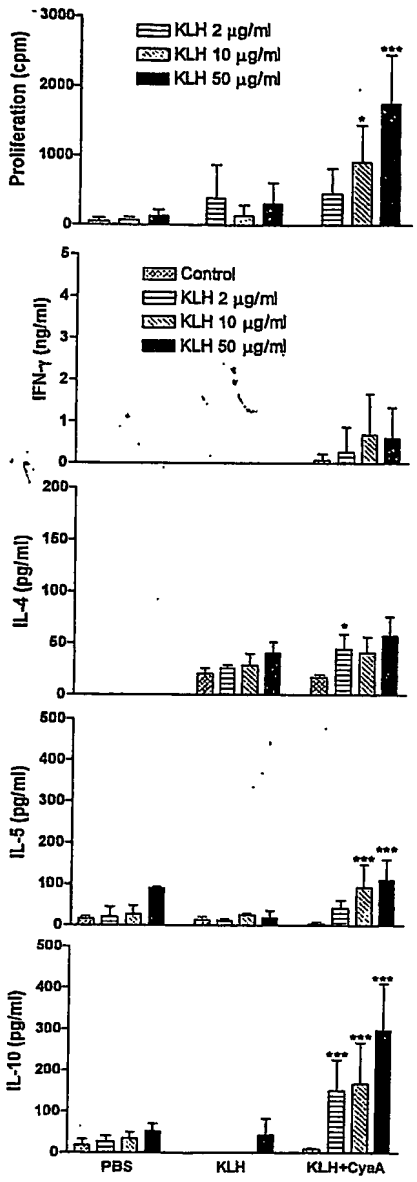
30. A vaccine comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

31. Antibodies to adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

32. Use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide or product of cells activated by the agent for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder.

33. Use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or variant or peptide or product of cells activated by the agent for the prophylaxis and/or treatment of diseases or conditions involving Toll-like receptor (TLR) dependant signalling.

34. Use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide or product of cells activated by the agent for the prophylaxis and/or treatment of asthma or allergy.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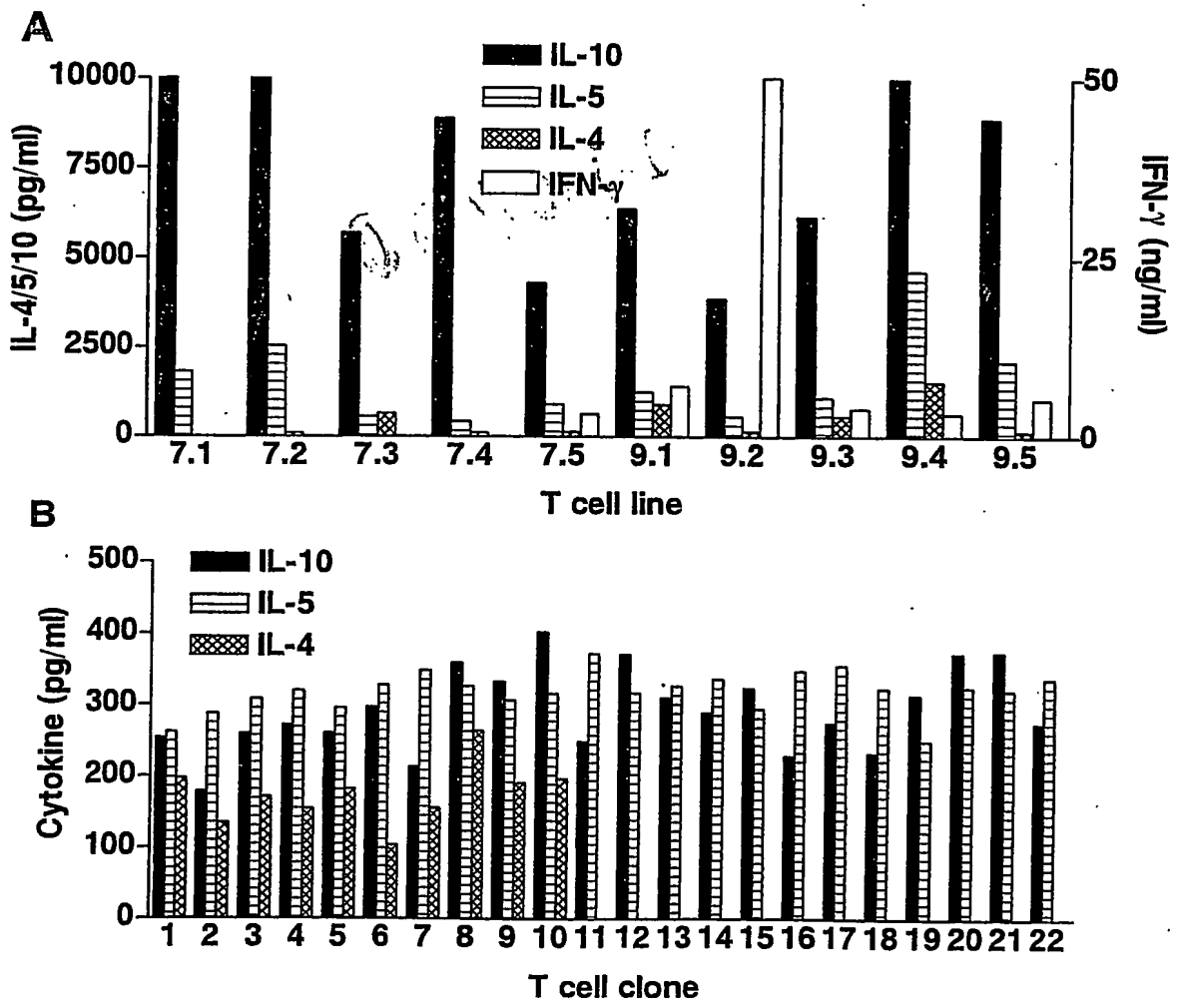
KLH 10 µg/ml
KLH 50 µg/ml
IFN-γ (ng/ml)
Control
KLH 2 µg/ml
KLH 10 µg/ml
KLH 50 µg/ml
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-10 (pg/ml)
PBS
KLH
KLH+CyaA

Fig. 1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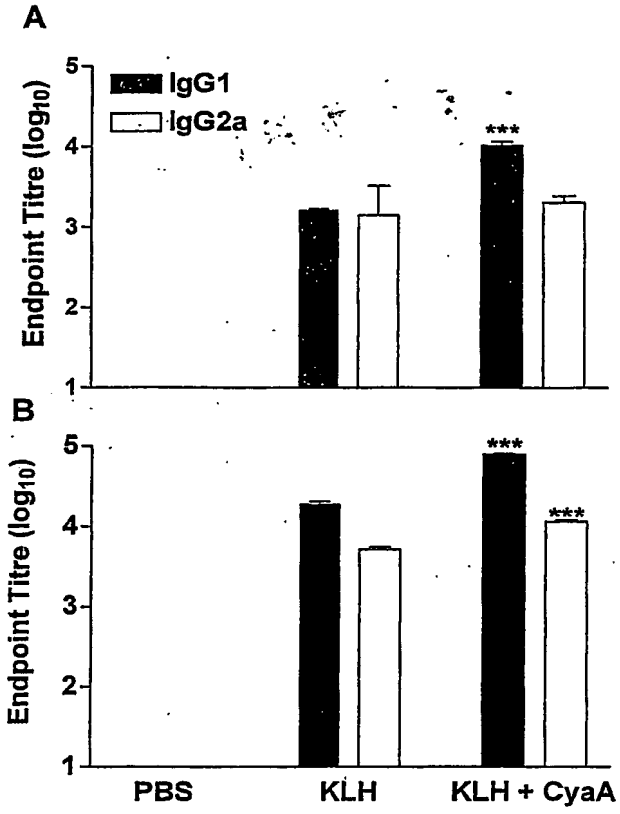
IL-5
IL-4
IFN-γ
10000
7500
5000
2500
0
IL-4/5/10 (pg/ml)
50
25
0
IFN-γ (ng/ml)
7.1
7.2
7.3
7.4
7.5
9.1
9.2
9.3
9.4
9.5
T cell line
B
IL-10
IL-5
IL-4
500
400
300
200
100
0
Cytokine (pg/ml)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
T cell clone

Fig. 2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IgG1
IgG2a
Endpoint Titre ($log_{10}$)
5
4
3
2
1
***
B
Endpoint Titre ($log_{10}$)
5
4
3
2
1
***
***
PBS
KLH
KLH + CyaA

Fig. 3

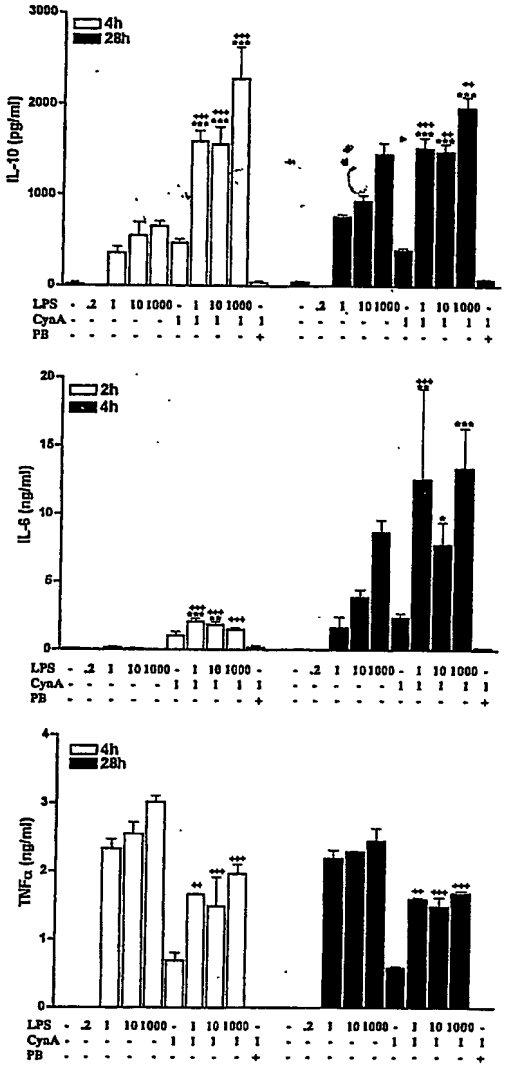
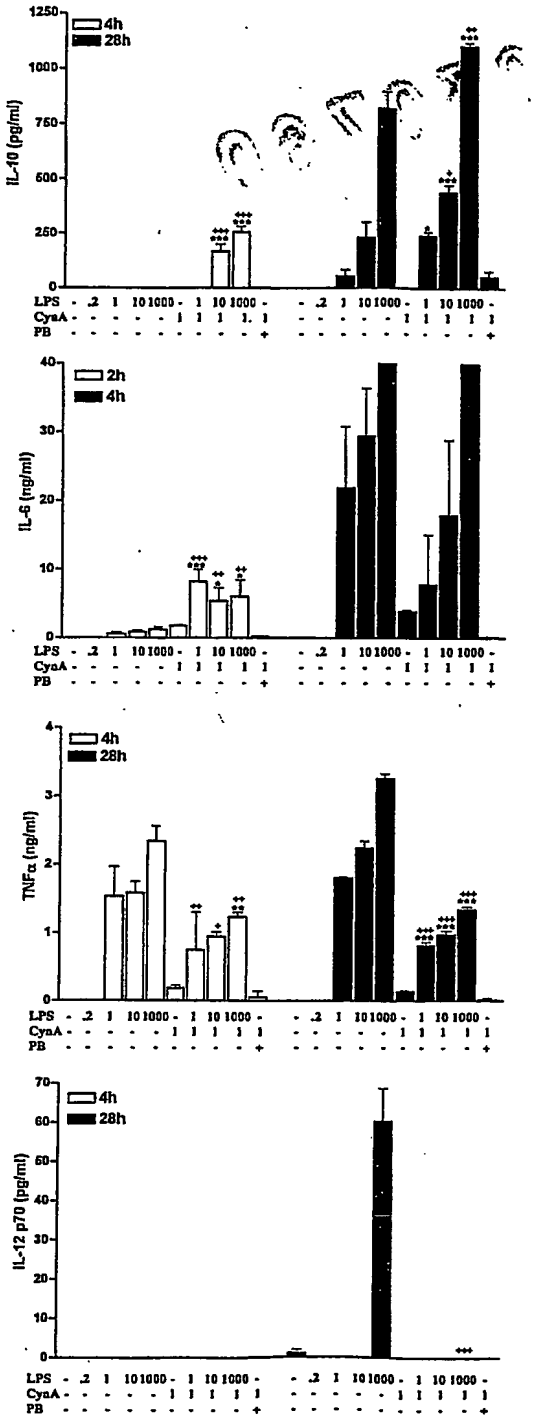

4h
28h
IL-10 (pg/ml)
3000
2000
1000
0
LPS - .2 1 10 1000 - 1 10 1000 - - .2 1 10 1000 - 1 10 1000 -
CynA - - - - - 1 1 1 1 1 - - - - - 1 1 1 1 1
PB - - - - - - - - - + - - - - - - - - - +
2h
4h
IL-6 (ng/ml)
20
15
10
5
0
LPS - .2 1 10 1000 - 1 10 1000 - - .2 1 10 1000 - 1 10 1000 -
CynA - - - - - 1 1 1 1 1 - - - - - 1 1 1 1 1
PB - - - - - - - - - + - - - - - - - - - +
4h
28h
TNFα (ng/ml)
4
3
2
1
0
LPS - .2 1 10 1000 - 1 10 1000 - - .2 1 10 1000 - 1 10 1000 -
CynA - - - - - 1 1 1 1 1 - - - - - 1 1 1 1 1
PB - - - - - - - - - + - - - - - - - - - +

**Fig. 4**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28h
IL-10 (pg/ml)
1250
1000
750
500
250
0
LPS - .2 1 10 1000 - 1 10 1000 -
CysA - - - - - 1 1 1 1 1
PB - - - - - - - - - +
2h
4h
IL-6 (ng/ml)
40
30
20
10
0
TNFα (ng/ml)
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3
2
1
IL-12 p70 (pg/ml)
70
60
50
40
30
20
10

Fig. 5

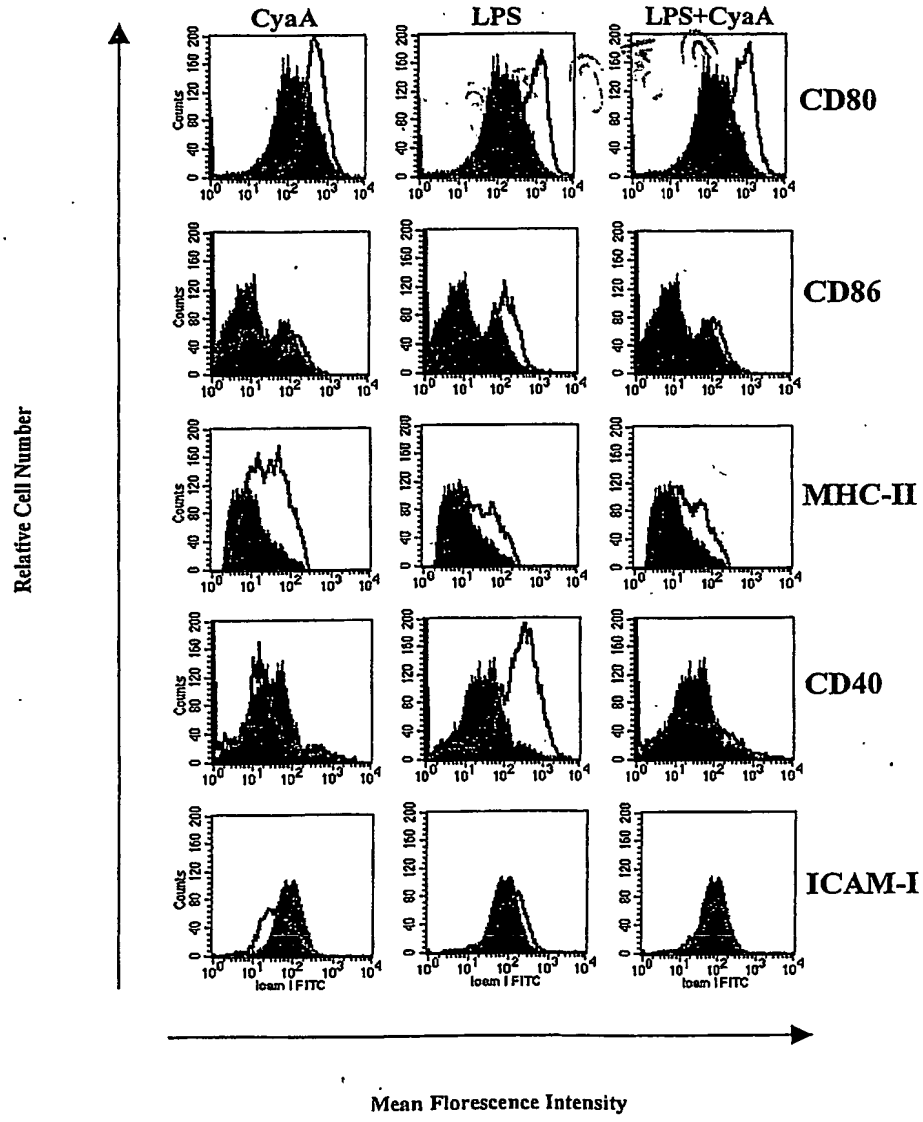
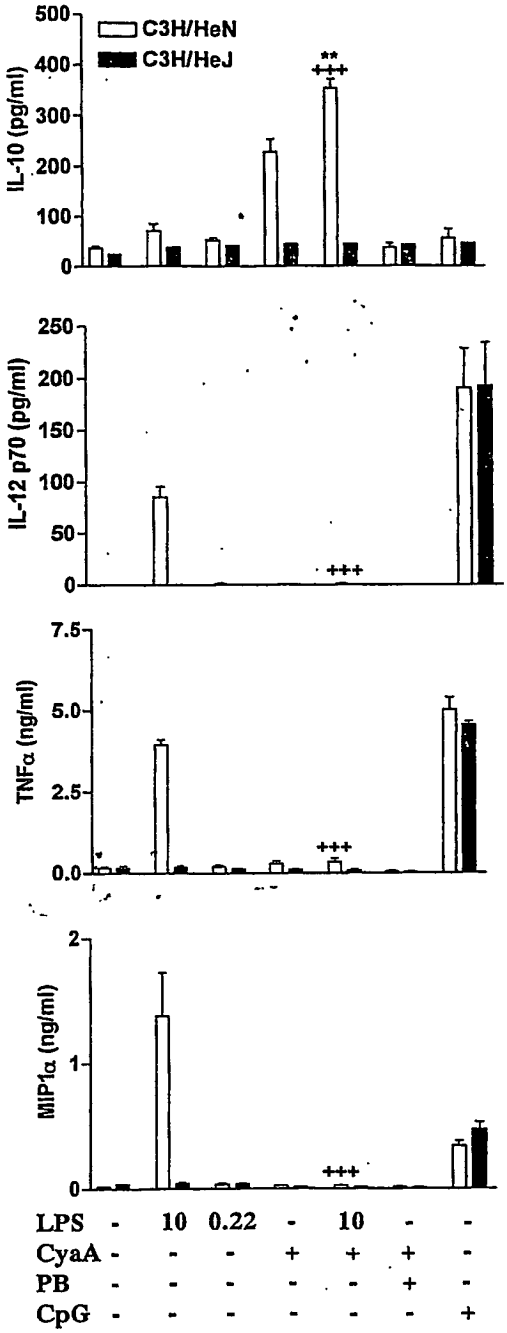

CyaA
LPS
LPS+CyaA
CD80
CD86
MHC-II
CD40
ICAM-I
Counts
Icam1 FITC
Relative Cell Number
Mean Florescence Intensity

Fig. 6


C3H/HeN
C3H/HeJ
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500
400
300
200
100
0
**
+++
IL-12 p70 (pg/ml)
250
200
150
100
50
0
+++
TNFα (ng/ml)
7.5
5.0
2.5
0.0
+++
MIP1α (ng/ml)
2
1
0
+++
LPS - 10 0.22 - 10 - -
CyaA - - - + + + -
PB - - - - - + -
CpG - - - - - - +

Fig. 7

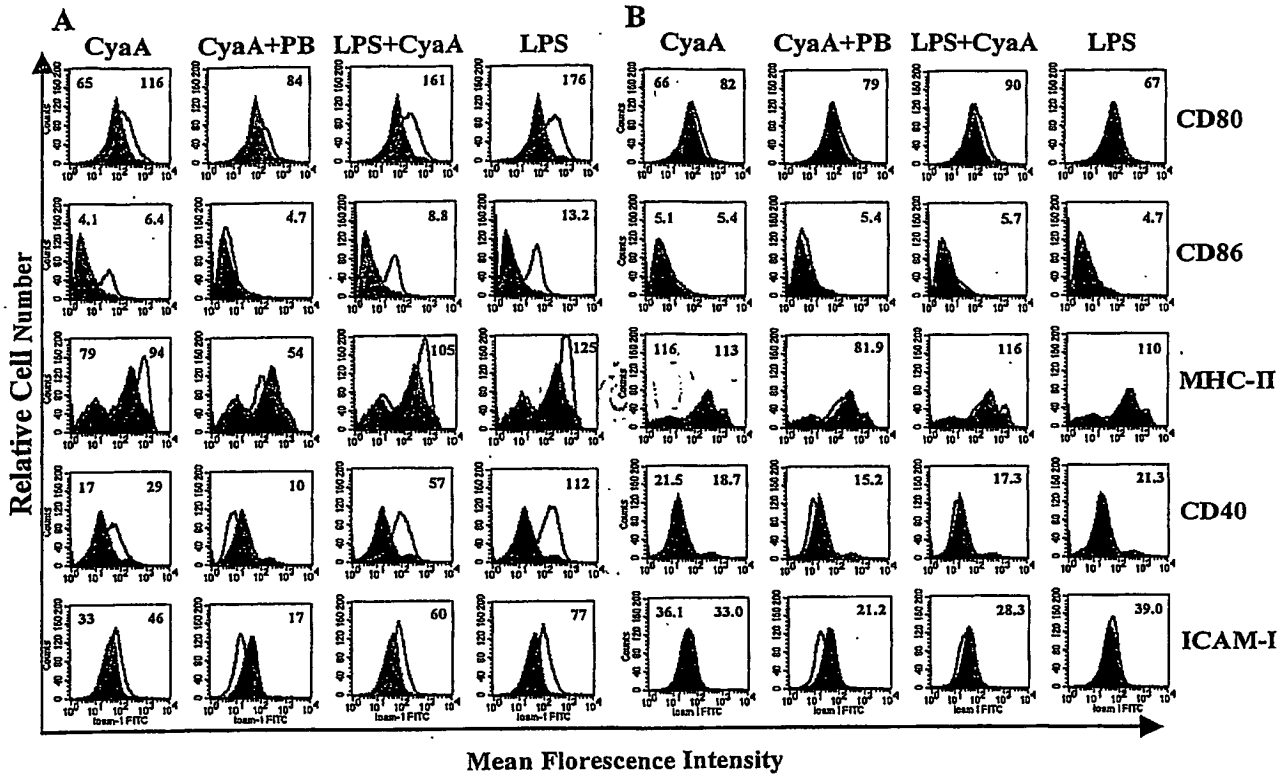

A
CyaA
CyaA+PB
LPS+CyaA
LPS
B
CyaA
CyaA+PB
LPS+CyaA
LPS
CD80
CD86
MHC-II
CD40
ICAM-I
Relative Cell Number
Mean Florescence Intensity

Fig. 8

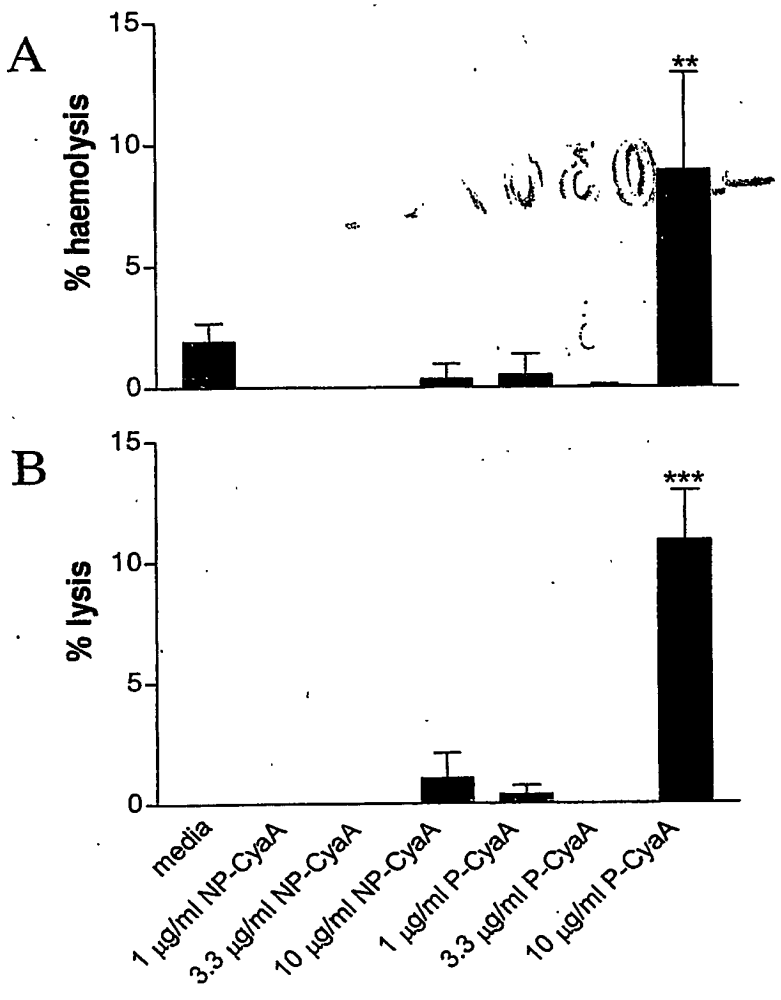
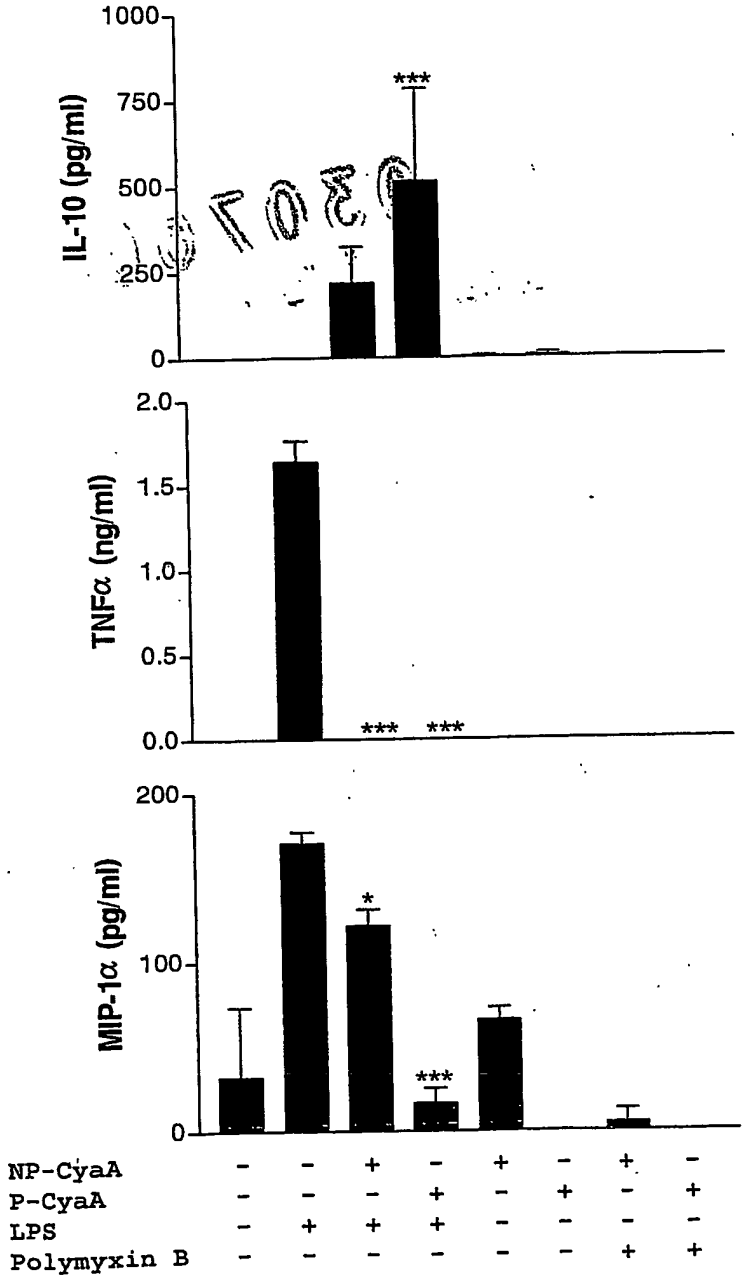

A
% haemolysis
15
10
5
0
**
B
% lysis
15
10
5
0
***
media
1 µg/ml NP-CyaA
3.3 µg/ml NP-CyaA
10 µg/ml NP-CyaA
1 µg/ml P-CyaA
3.3 µg/ml P-CyaA
10 µg/ml P-CyaA

Fig. 9

Fig. 10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1000
750
500
250
0
***
TNFα (ng/ml)
2.0
1.5
1.0
0.5
0.0
*** ***
MIP-1α (pg/ml)
200
100
0
*
***
NP-CyaA - - + - + - + -
P-CyaA - - - + - + - +
LPS - + + + - - - -
Polymyxin B - - - - - - + +

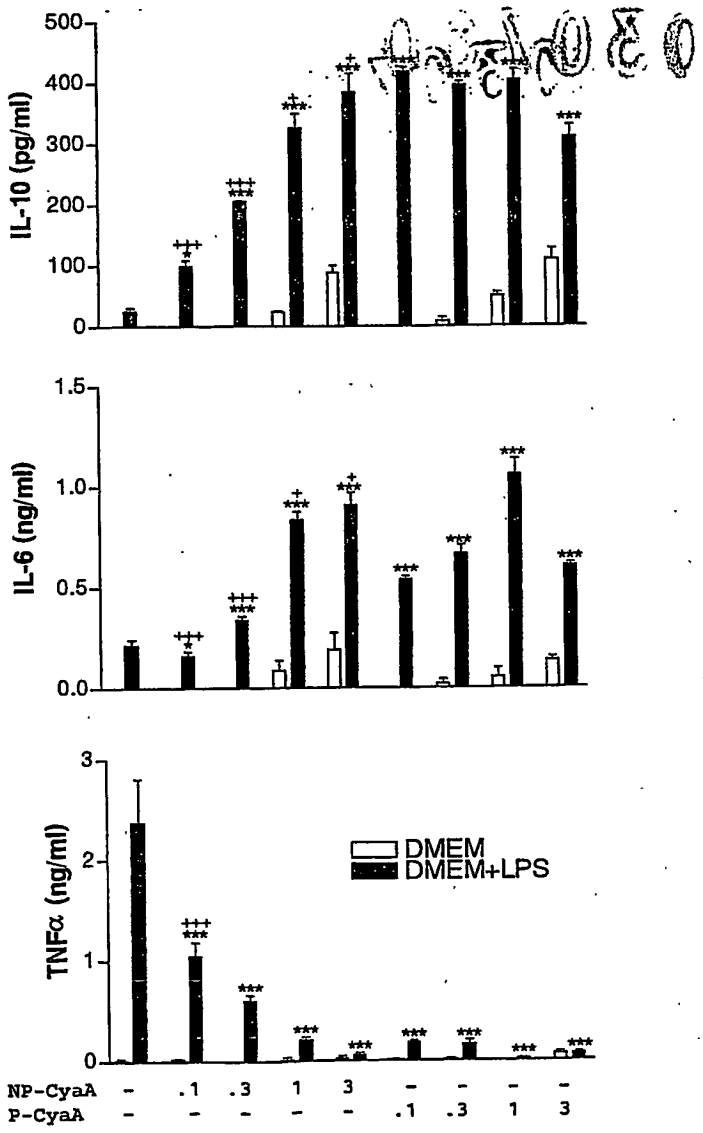
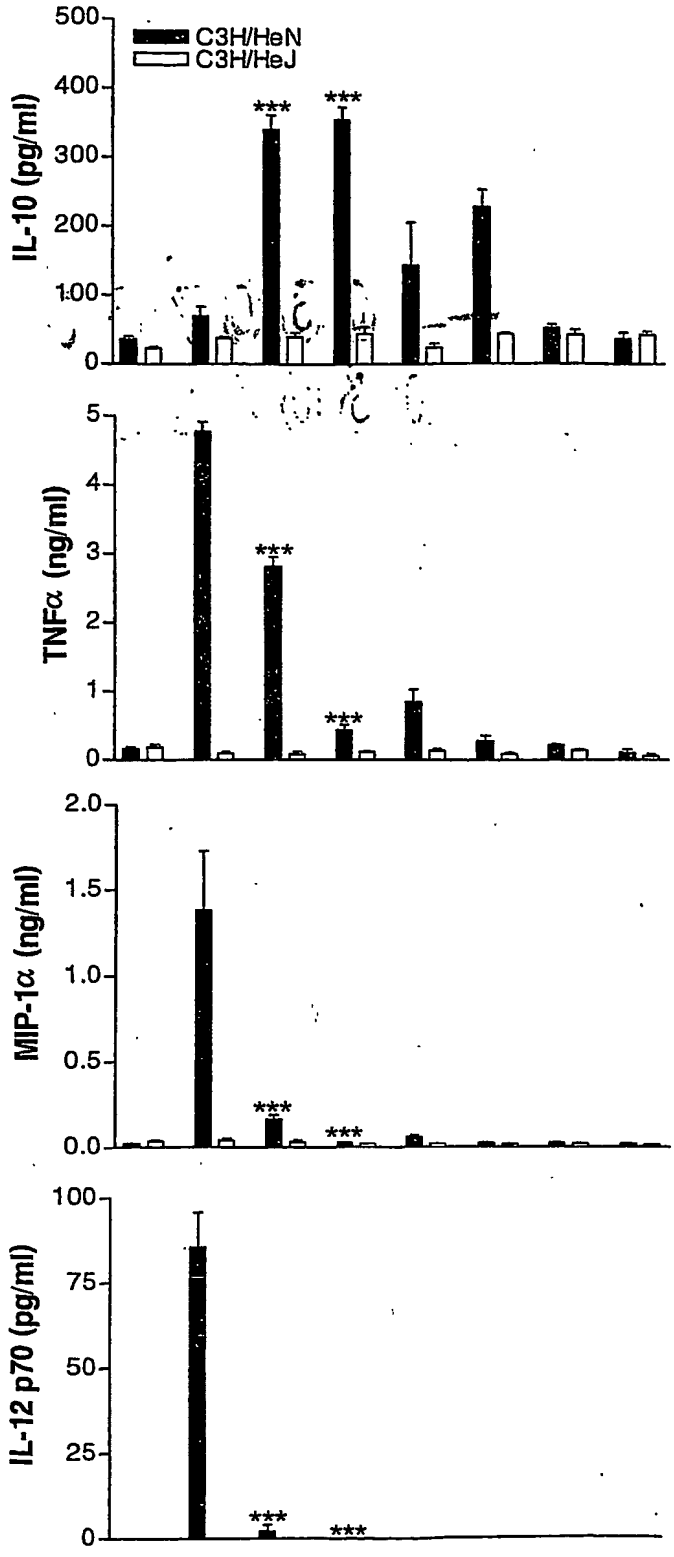

IL-10 (pg/ml)
500
400
300
200
100
0
IL-6 (ng/ml)
1.5
1.0
0.5
0.0
TNFα (ng/ml)
3
2
1
0
DMEM
DMEM+LPS
NP-CyaA - .1 .3 1 3 - - - -
P-CyaA - - - - - .1 .3 1 3

Fig. 11

Fig. 12


C3H/HeN
C3H/HeJ
IL-10 (pg/ml)
500
400
300
200
100
0
***
***
TNFα (ng/ml)
5
4
3
2
1
0
***
***
MIP-1α (ng/ml)
2.0
1.5
1.0
0.5
0.0
***
***
IL-12 p70 (pg/ml)
100
75
50
25
0
***
***
NP-CyaA - - + - + - + -
P-CyaA - - - + - + - +
LPS - + + + - - - -
Polymyxin B - - - - - - + +

# Fig. 13

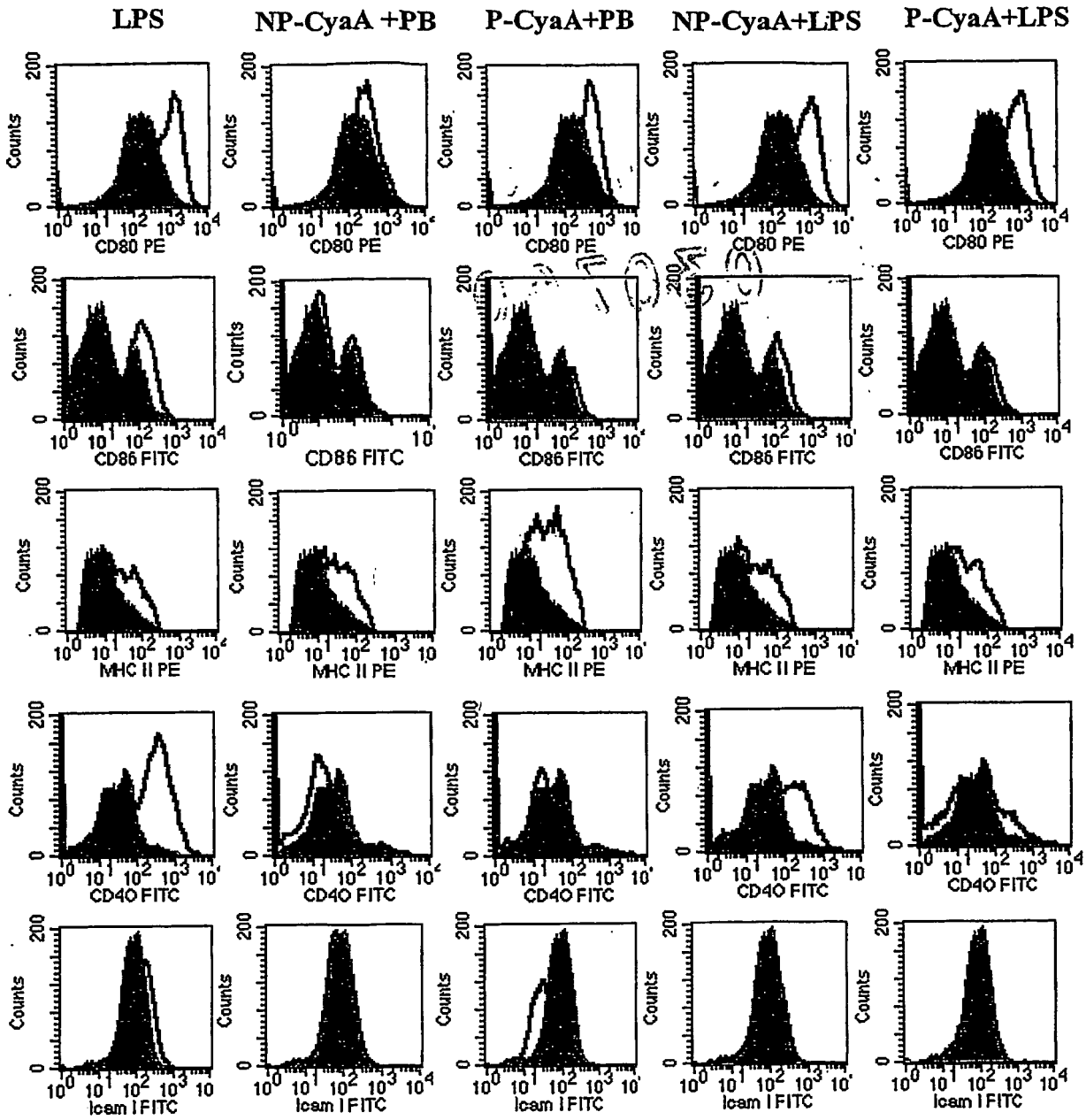
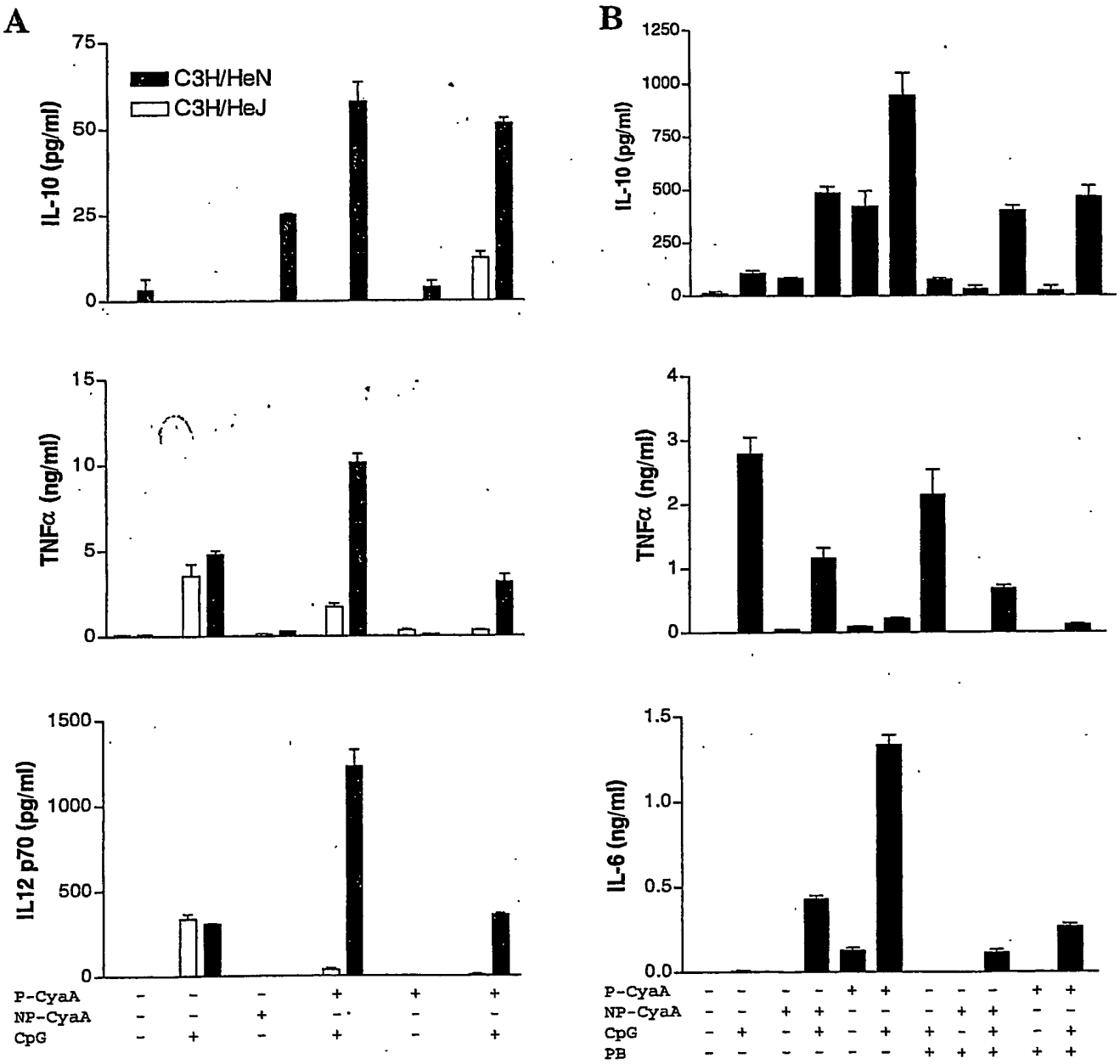
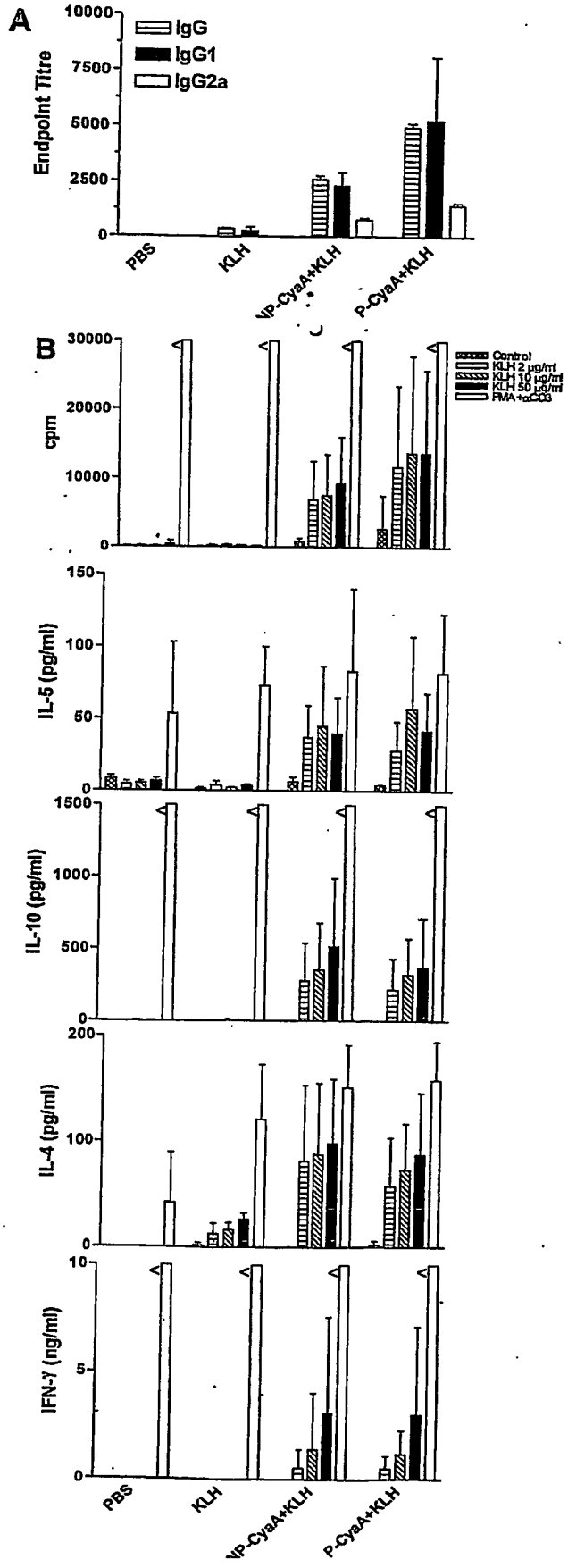

LPS
NP-CyaA +PB
P-CyaA+PB
NP-CyaA+LPS
P-CyaA+LPS
Counts
CD80 PE
CD86 FITC
MHC II PE
CD40 FITC
Icam I FITC

Fig. 14


A
B
C3H/HeN
C3H/HeJ
IL-10 (pg/ml)
TNFα (ng/ml)
IL12 p70 (pg/ml)
IL-6 (ng/ml)
P-CyaA
NP-CyaA
CpG
PB


A
Endpoint Titre
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7500
5000
2500
0
IgG
IgG1
IgG2a
PBS
KLH
VP-CyaA+KLH
P-CyaA+KLH
B
cpm
30000
20000
10000
0
Control
KLH 2 µg/ml
KLH 10 µg/ml
KLH 50 µg/ml
PMA+αCD3
IL-5 (pg/ml)
150
100
50
0
IL-10 (pg/ml)
1500
1000
500
0
IL-4 (pg/ml)
200
100
0
IFN-γ (ng/ml)
10
5
0
PBS
KLH
VP-CyaA+KLH
P-CyaA+KLH

Fig. 15

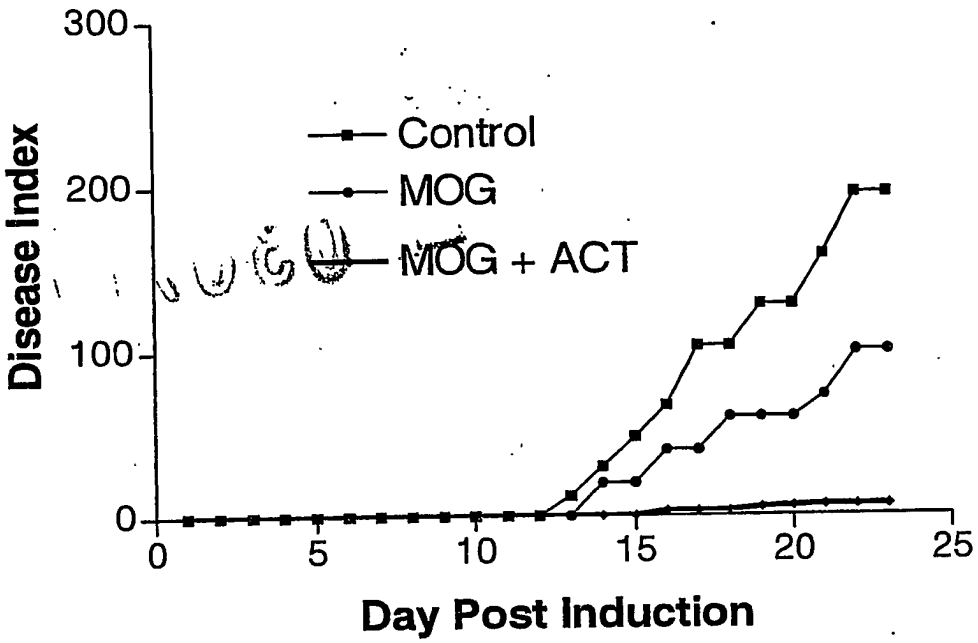
300
200
100
0
Disease Index
Control
MOG
MOG + ACT
0
5
10
15
20
25
Day Post Induction

Fig. 16

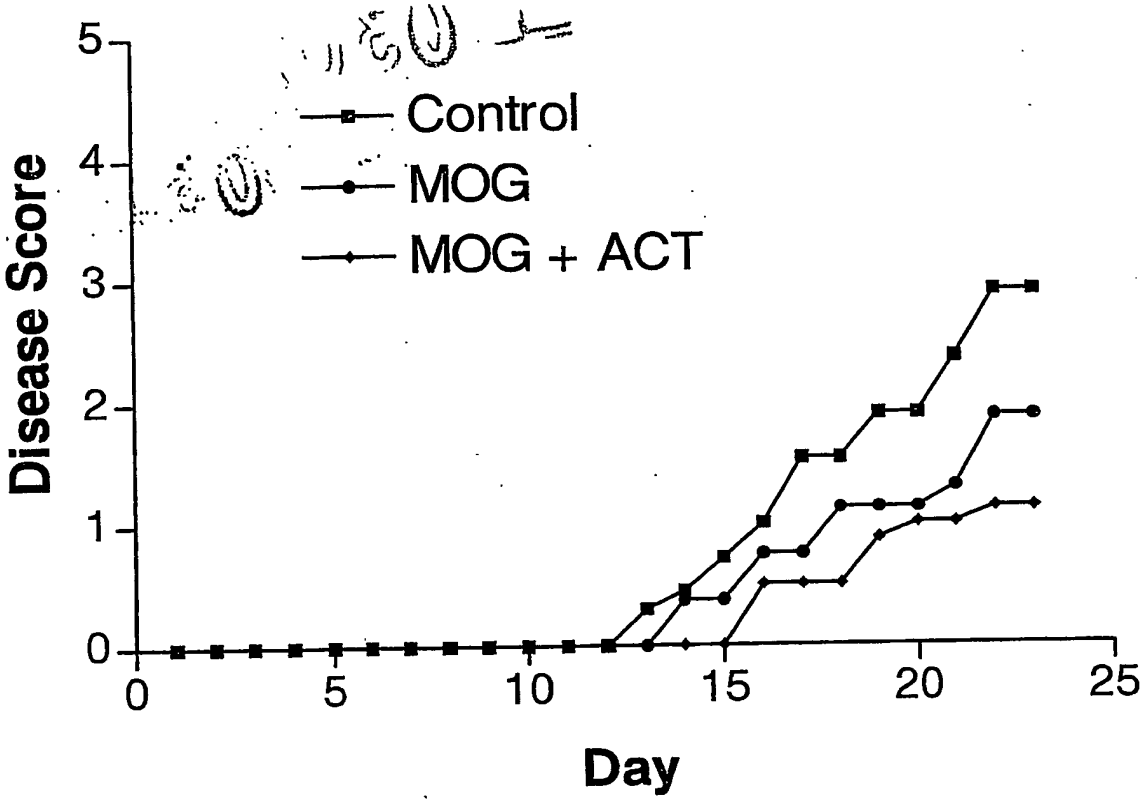


Fig. 17

# Immunization with MOG and CyA prevents brain encephalitis associated with the induction of EAE

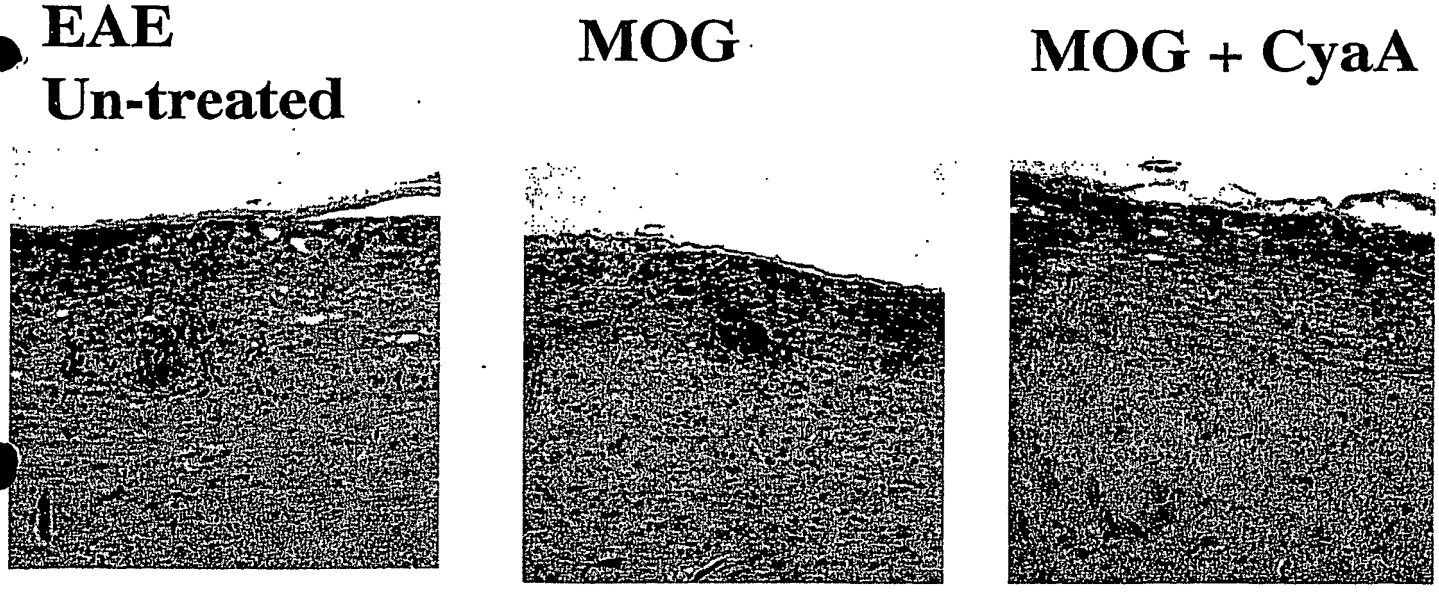


Fig. 18